The Glucose-6-phosphate dehydrogenase encoding genes from *Aspergillus niger* and *Aspergillus nidulans*

Peter van den Broek

Proefschrift

Ter verkrijging van de graad van doctor

Op gezag van de rector magnificus,

van de Landbouwuniversiteit Wageningen,

dr. C.M. Karssen, in het openbaar te verdedigen

op vrijdag 21 november 1997 des namiddags vier uur

in de Aula

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Stellingen

The observation that a G6PD-null mutant of *Saccharomyces cerevisiae* still exhibits carbon catabolite repression, does not mean that this repression is not exerted by an intermediate from the pentose phosphate pathway.
(Nogae, I. and Johnston, M. (1990) Isolation and characterization of the *zwf1* gene of *Saccharomyces cerevisiae*, encoding glucose-6-phosphate dehydrogenase. Gene 96: 161-169).

The statement by Nogae and Johnstone, that G6PD expression in *S. cerevisiae* is not regulated is contradicted by their own results.
(Nogae, I. and Johnston, M. (1990) Isolation and characterization of the *zwf1* gene of *Saccharomyces cerevisiae*, encoding glucose-6-phosphate dehydrogenase. Gene 96: 161-169).

Alignment of 5' proximal regions of protein encoding genes as a tool for identification of potential *cis*-acting elements, yields only reliable data if the regulation of transcription of these genes is identical.
(Dit proefschrift)

Even the specific binding *in vitro* of the *N. crassa nit-2* gene product to sequences in the *Lycopersicon esculentum* nitrate reductase (*nia*) promoter, does not prove that a *nit2* homologue exerts nitrogen metabolite repression in tomato.
Jarai, G., Truong, H. Daniel-Vedele, F. and Marzluf, G.A. (1992) Nit-2, the nitrogen regulatory protein of *Neurospora crassa* binds upstream of *nia*, the tomato nitrate reductase gene, in vitro. Curr. Genet. 21: 37-41.

The high level of transcription of human G6PD-CAT reporter construct, observed by Ursini and coworkers, could be an artefact from their transient expression assay. (Ursini, M.V., Scalera, L. and Martini, G. (1990) High levels of transcription by a 400 bp segment of the human G6PD promoter. Biochem. Biophys. Res. Comm. 170: 1203-1209).

The statement that a *Drosophila melanogaster Zw null* mutant has no phenotype is premature. (Gvozdev, V.A., Gerasimova, T.I., Kogan, G.L. and Rosovsky, J.M. (1977) Investigations on the organization of genetic loci in *Drosophila melanogaster*: lethal mutations affecting 6-phosphogluconate dehydrogenase and their suppression. Mol. Gen. Genet. 153: 191-198)

The observation that two different translational fusions of a gene with the *lac*Z reporter gene differ in their ß-galactosidase activities, can not be taken as proof for the presence of *cis*-acting elements within the coding region. (Streatfield, S.J., Toews, S. and Robberts, C.F. (1992) Functional analysis of the expression of the 3'-glycerate kinase *pgk* gene in *Aspergillus nidulans*. Mol. Gen. Genet. 233: 231-240.)

Zeilen is een technisch hoog ontwikkelde uiting van hydrofobie.
The data, cited by Berbesgaard and co-workers to argue the safety of *Aspergillus oryzae* in food fermentations, actually give rise to serious concern. (Berbesgaard, P., Heldt-Hansen, H. and Diderichsen, B. (1992) On the safety of *Aspergillus oryzae*: a review. Appl. Microbiol. Biotechnol. 36: 569-572.

Het is onterecht om schimmels, die zich niet sexueel voortplanten van het predicaat imperfect te voorzien.

De aanduiding NOPPER voor medewerkers, die met behoud van uitkering aan de LU onderzoeks ervaring opdoen, wekt geheel ten onrechte de indruk dat deze mensen nutteloos werk verrichten.

Gezien hun blootstelling aan hoge partiele O2 drukken, is het bezit van een wild-type G6PD allel voor duikers van levensbelang.

Stellingen behorend bij het proefschrift "Glucose-6-phosphate-dehydrogenase encoding genes from *Aspergillus niger* and *Aspergillus nidulans*."

Wageningen, 21 november 1997
Peter van den Broek

Contents

Abbreviations

ATP	adenosine-5'-triphosphate
ADP	adenosine-5'-diphosphate
BSA	bovine serum albumin
CM	complete medium
DEPC	diethylpyrocarbonate
dNTP	dideoxynucleotide triphosphates
E4P	erythrose-4-phosphate
EDTA	ethylenediamine tetra acetic acid
F6P	fructose-6-phosphate
G3P	glyceraldehyde-3-phosphate
G6P	glucose-6-phosphate
G6PD	D-glucose-6-phosphate:NADP+ oxidoreductase (EC 1.1.1.49)
Ki	inhibition constant
Km	Michaelis constant
MCA	Metabolic Control Analysis
MM	minimal medium
MOPS	morpholinopropanesulphonic acid
NAD/NADH	nicotinamide dinucleotide
NADP/NADPH	nicotinamide dinucleotide phosphate
NADP-ICD	isocitrate:NADP+ oxidoreductase (decarboxylating) (EC 1.1.1.42)
NADP-ME	(S)-malate:NADP+ oxidoredutase (EC 1.1.1.40)
NADP-ALD	alditol:NADP+ 1-oxidoreductase (EC 1.1.1.21)
OAA	oxaloacetic acid
ONPG	ortho-nitrophenyl-ß-D-galactopyranoside
PAA	polyacrylamide
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEP	phosphoenolpyruvic acid
6PGD	6-phospho-D-gluconate:NADP+ 2-oxidoreductase (EC 1.1.1.44)
PMSF	phenyl-methyl-sulfonyl fluoride
R5P	ribose-5-phosphate
Ru5P	ribulose-5-phosphate
S7P	sedoheptulose-7-phosphate
SDS	sodium dodecylsulphate
SMM	supplemented minimal medium
SSC	saline sodium citrate buffer
TRIS	tris(hydroxymethyl)aminomethane
TSP	transcription start point
UAS	upstream activating sequence
URS	upstream repressing sequence
WT	wild type
X-GAL	5-bromo-4-chloro-3-indoyl-ß-galactopyranoside

Voorwoord

Het heeft heel lang geduurd, maar nu is het boekje dan eindelijk af! Dat dit proefschrift er toch gekomen is, vloeit voort uit één van de opvoedingsprincipes van mijn ouders "je maakt af, wat je begonnen bent". Voor die opvoeding, de kans om te gaan studeren en hun steun wil ik mijn vader en moeder hier bedanken.

Theo, het verschil in temperament tussen ons heeft wel eens aanleiding gegeven tot spanningen in het lab. Het blijft echter een feit dat zonder jouw raad en persoonlijke inzet dit proefschrift er nooit gekomen zou zijn. Het feit dat ik bij mijn huidige werkgever in staat was een Aspergillus lab op te zetten, zegt eigenlijk genoeg over de scholing door jou en Henk. Henk, hoe vaak heb jij moeten uitleggen dat je niet mijn vader was? Wat mij betreft is dat na vandaag opgelost; vanwege de vaderlijke begeleiding en de aansporende E-mails naar Zwitserland verdien jij de titel "Doctorvater". Bert, bedankt voor alle experimenten die je voor mij hebt uitgevoerd en voor je onverwoestbare optimisme dat alles goed zou komen. Ook mijn studenten, Monique Jacobs, Karin Eizema, Jan-Albert Kuivenhoven, Mirjam Goedbloed, Hein Boot, Eric Buitenhuis, Michiel Hellendoon, Michiel van Hoof, Annouschka Glas, Elise Bal, Pieter van Santen, Dirk Ruiter, Frauwke Hangen, Kerstin Schommer, Conny Mol, Arjen ten Have, Maurice Jansen en Wilco Ligterink hebben met enthousiasme veel werk verzet voor dit boekje.

Jaap Visser en Peter Schaap zou ik willen danken voor het her-kloneren en her-sequencen van het *A. nidulans gsd*A gen. Op aanvraag waren onze *A. nidulans gsd*A klonen ook voor hen beschikbaar geweest.

Mijn promoter Professor Christa Heyting dank ik voor het kritisch doorlezen van het proefschrift en de ondersteuning bij de laatste loodjes. Jan van der Poel, Martin Groenen en Rosilde Dijkhof van de vakgroep Veefokkerij verdienen in dit voorwoord een ereplaats vanwege de ondersteuning bij de sequentie analyse en het 'lenen' van het nodige 35S-label. Ook Cees van den Hondel en Peter Punt hebben door de snelheid waarmee ze ons van informatie, stammen en plasmieden voorzagen een belangrijke bijdrage geleverd aan dit proefschrift. De gastmedewerkers Roland Marmeise, Guido van der Ackerveken, Rado Valinger, Jane Newbury en Kees Langeveld bedank ik hun collegialiteit en de gezelligheid op het lab. Het gezelschap van Jan van Kan tijdens de NS-half uurtjes (in het gunstigste geval!) Ede-Utrecht maakte het forensen een stuk aangenamer. Ook mijn huidige chef Peter Niederberger wil ik hier graag bedanken, omdat hij toeliet dat ik een post-doc imiteerde.

Dr Annette Scherpenzeels' bijdrage aan dit boekje is zelfs met beste statistische software niet te schatten en ik dank haar voor het geven van het goede voorbeeld, de vele knuffeltjes, de praktische (het schema!) en morele steun. Na 21 november 1997 hoef ik dan alleen nog mijn rijbewijs te halen om de gelijkwaardigheid in de relatie te herstellen. Ons mannetje Niels heeft er nog geen weet van, maar zijn komst was wel de aanleiding om "het boekje" eindelijk af maken.

Peter van den Broek
Epalinges, Augustus 1997

Chapter 1 General introduction

Filamentous fungi, especially *Aspergillus nidulans*, *Aspergillus niger* and *Neurospora crassa*, have been extensively used as model systems to study the regulation of metabolic pathways, eukaryotic gene organisation and regulation of gene expression. In the following chapters, the metabolic versatility and the amenability to genetic manipulation of *A. nidulans* and *A. niger* have been exploited to study the regulation of expression of the gene for glucose-6-phosphate dehydrogenase (G6PD). G6PD catalyses the first and committing reaction of the pentose phosphate pathway. This pathway can be divided in an oxidative part which produces NADPH and a non-oxidative part that generates four-, five-, six- and seven-carbon sugars. In biochemistry textbooks, the oxidative branch of pentose phosphate pathway is usually considered to be the primary source for cytosolic NADPH. This NADPH acts as donor of reduction equivalents in a large number of biosynthetic reactions. However, several studies seem to challenge the view that the two oxidative reactions in the pentose phosphate pathway are the major suppliers of NADPH. In an attempt to clarify this issue, we chose to study the regulation of expression of the gene encoding G6PD from *A. niger* and *A. nidulans* in response to changes in NADPH consumption.

The Aspergilli

The genus *Aspergillus* is a group of filamentous fungi that derives its name from the characteristic form of its sporeheads and conidiphores, reminiscent of the mop used to sprinkle holy water in the catholic church (Lat. *aspergillium*) (Raper and Fennel, 1965).

The sophisticated genetics, the large number of well characterised mutants and the availability of a DNA-mediated transformation system for *A. nidulans* (Ballance, 1983) helped to establish this species as a model system for the study of the regulation of gene expression in lower eukaryotes. The progress in this particular field has been such, that current research efforts aim to use several *Aspergillus* species as heterologous expression systems (Ward, 1991).

Because of its ability to grow on a wide variety of substrates, *A. nidulans* is also an ideal model system to study metabolism (Uitzetter, 1982). The metabolic versatility of other members of the genus, especially of the black Aspergilli (e.g. *A. niger* and *A. awamori*), is exploited in the industrial production of organic acids like: citric acid, itaconic acid, gallic acid and gluconic acid. Also for the commercial production of enzymes like α-amylase, glucoamylase, catalase, glucose oxidase, lactase, ß-galactosidase, lipases, proteases and pectinolytic enzymes several members of the genus *Aspergillus* are used (Elander and Lowe, 1992). From ancient times both members of the black Aspergilli (i.e. *A. niger* and *A. awamori*) and several members of the yellow Aspergilli (i.e. *A. oryzae* and *A. sojae*) have been used in oriental food fermentation (soy sauce and miso) and preparation of alcoholic beverages (saké and jiu) (Sakaguchi *et al.*, 1992).

The genus *Aspergillus* includes also a number of harmful species. Some species, like *A. flavus*, *A. parasiticus* and *A. ochraceus*, can produce powerful mycotoxins (e.g. aflatoxins, ochratoxin and cyclopiazonic acid). Other species like *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus* can cause aspergillosis: an invasive infection of the respiratory tracts in man and animals (for review see Dixon and Walsh, 1992).

According to the rules of the International Code of Botanical Nomenclature (ICBN), *Aspergillus* is an anamorphic genus. This means that true Aspergilli only reproduce asexually through conidiospores

(Samson, 1992). However, the typical *Aspergillus* conidiophore morphology can also be found in fungi that can reproduce sexually via ascospores (e.g. *Aspergillus nidulans*). Some Aspergillus taxonomists caused confusion, because they did not adhere to ICBN terminology. Instead, they attempted to make various revisions of taxonomical schemes to include, among others, *A. nidulans* in this genus. Since *Emericella nidulans* has been used for genetic and biochemical analysis under the name of *Aspergillus nidulans* for over hundred years, its taxonomically correct name is rarely used. To avoid further confusion, several fungal taxonomists advocate the use of well established names instead of taxonomically correct names (for example *Aspergillus nidulans* instead of *Emericella nidulans*) (Samson, 1992). In accordance with this proposition, throughout the following chapters, *E. nidulans* will be referred to as *A. nidulans*. Despite their assignment to different genera, strong sequence conservation in the 18S rRNA genes (Verweij *et al.*, 1995), mitochondrial DNA (Kozlowski and Stepien, 1982) and protein encoding genes (e.g. *pyr*G and *nia*D) suggests that *A. niger* and *A. nidulans* are closely related.

NADPH, the reduction equivalent in anabolic metabolism

To sustain anabolic metabolism, the catabolic metabolism in a cell has to meet three basic requirements: the production of sufficient energy, precursors and reductive power. Energy is supplied in the form of ATP, generated either by oxidative phosphorylation, dissipating the proton motive force, or by substrate phosphorylation. Intermediates from catabolic pathways serve as precursors for anabolic metabolism. In general the NADPH/$NADP^+$ redox couple acts as a donor of reduction equivalents in biosynthetic reactions (see Klingenberg and Bücher, 1960) while the NADH/NAD^+ redox couple acts as electron acceptor in catabolic metabolism (Lehninger, 1951). The observation in *Saccharomyces cerevisiae* (Saez and Lagunas, 1976), bacteria (Voordouw *et al.*, 1983) and mammals (Sies, 1982), that the NADPH/$NADP^+$ redox couple is in a more reduced state than the NADH/NAD^+ redox couple, would be consistent with their distinct roles in cellular metabolism. Even in organisms or organelles that possess a transhydrogenase activity, which would allow exchange of hydrogen atoms between NADP(H) and NAD(H), such a difference in reduction level between the two dinucleotide redox couples has been observed (Sies, 1982; Voordouw *et al.*, 1983).

NADPH consuming reactions in *A. nidulans* and *A. niger*

In most organisms all of the reductive steps in the biosynthesis of amino acids and nucleic acids are catalysed by NADPH-dependent enzymes. Also the biosynthesis of fatty acids from acetyl-CoA is a major NADPH consuming process, because both ß-ketoacyl-ACP-reductase and enoyl-ACP-reductase in fungi are NADPH-specific (Walker and Woodbine, 1976). A third process that consumes considerable amounts of NADPH is nitrate assimilation, because nitrate reductase and nitrite reductase in *A. nidulans* (Cove, 1979) and *A. niger* (Unkles *et al.*, 1992) are strictly NADPH-dependent.

The pentoses D-xylose and L-arabinose are converted to ribulose-5-phosphate in *A. niger*. Since this conversion to ribulose-5-phosphate involves reduction by NADPH-linked pentitol reductases and oxidation by NAD^+-linked pentose oxidases, growth on these carbon sources requires net input of NADPH (Bruinenberg *et al.*, 1983; Witteveen *et al.*, 1990).

In other organisms additional NADPH consuming processes have been described; for example detoxification of superoxide radicals by superoxide dismutase and peroxides by catalase (Greenberg and Demple, 1991). Also the intracellular glutathione pool, which is the first line of defence against oxidation damage, depends on a NADPH-linked reductase for regeneration (Beutler, 1986).

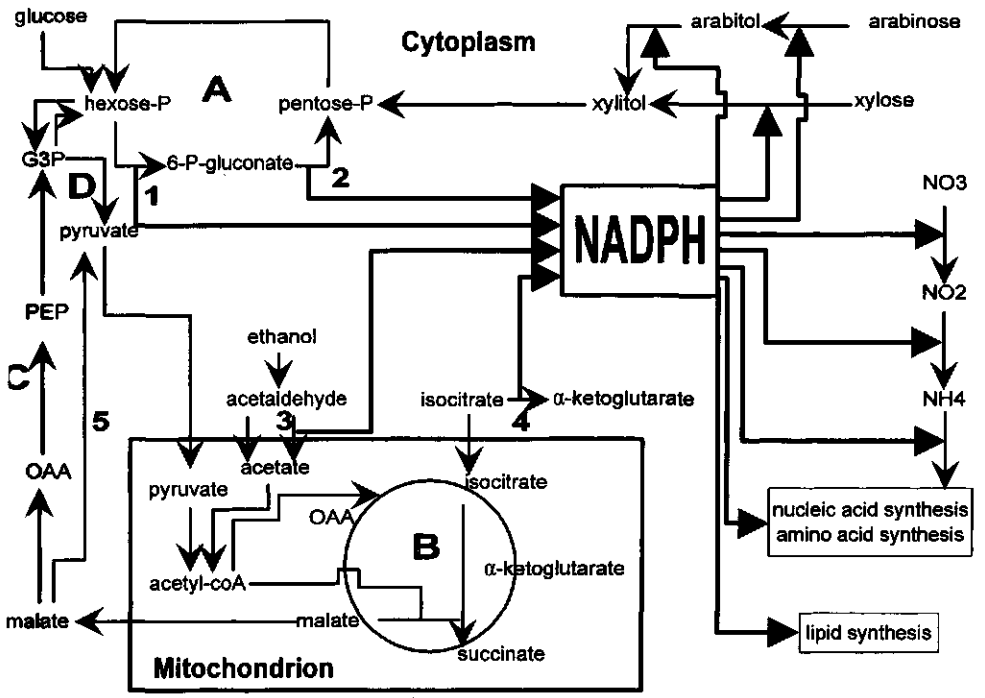

Figure 1. NADPH producing and consuming pathways in *A. nidulans*.

A simplified view of the NADPH generating and consuming pathways and their subcellular localisation adapted from Bruinenberg *et al.* (1982) for *A. nidulans* according to Mckorkindale (1976), Cochrane (1976), Casselton (1976), Uitzetter (1983), Kelly and Hynes (1981a/b) and Singh *et al.* (1988). In this scheme, it is assumed that D-xylose and L-arabinose are metabolised in *A. nidulans* as described for *A. niger* (Witteveen *et al.*, 1990). The reactions of the glyoxylate pathway, bypassing the decarboxylating reactions of the citric acid cycle, have been depicted as if localised in the mitochondrion, but they could also reside in a specialised microbody the glyoxysome. During growth on acetate both G6PD and $NADP^+$-linked ICD have been shown to contribute to NADPH production (Singh *et al.*, 1988). Under these conditions the glyoxylate cycle allows synthesis of glucose from acetyl-CoA, therefore this cycle is important for generation of NADPH during growth on acetate. Since no reports on energy dependent or energy independent NAD(P)-transhydrogenase in *Aspergillus* exist, these enzymes have not been included in the scheme. **Legend: OAA** = oxaloacetic acid, **PEP** = phosphoenolpyruvic acid and **G3P** = glyceraldehyde-3-phosphate. **A** = pentose phosphate pathway, **B** = citric acid cycle (tricarboxylic acid cycle)/glyoxylate cycle, **C** = gluconeogenetic pathway, **D** = glycolytic pathway, **1** = glucose-6-phosphate dehydrogenase (G6PD), **2** = 6-phosphogluconate dehydrogenase (6PGD), **3** = $NADP^+$-linked aldehyde dehydrogenase ($NADP^+$-ALD), **4** = NADP+-linked isocitrate dehydrogenase ($NADP^+$-ICD) **5** = NADP+-linked malate dehydrogenase ($NADP^+$-ME: Malic enzyme).

NADPH producing reactions in *A. nidulans* and *A. niger*

In heterotrophic organisms there are five major NADPH generating reactions (Fig. 1) which are catalysed by: glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), $NADP^+$-linked isocitrate dehydrogenase ($NADP^+$-ICD), $NADP^+$-linked malate dehydrogenase (malic enzyme, $NADP^+$-ME) and $NADP^+$-linked aldehyde dehydrogenase (ALD).

The contribution of these reactions to the total NADPH production depends on the regulation of these enzymes and the availability of their substrates. Furthermore, if mitochondrial inner membranes in Aspergilli are as impermeable for pyridine dinucleotides as those in *S. cerevisiae* (von Jagow and Klingenberg, 1970), not only the regulation of a NADPH-generating enzyme but also its subcellular localisation must be considered to asses the contribution of an enzyme to the NADPH production (Bruinenberg *et al.*, 1983a). Since most of the NADPH consuming reactions are localised in the cytoplasm, consequently also the major NADPH producing enzymes should reside in that compartment.

In *A. nidulans* data on the regulation of some of the NADPH generating enzymes are available as well as their subcellular localisation (Fig. 1). This allows an assessment of their contribution to the NADPH pool under different growth conditions. Despite the fact that *A. niger* is widely used for industrial production of organic acids and enzymes, information on both the presence as well as the regulation of the equivalent enzymes is scarce.

In *A. nidulans* mycelium grown on glucose, glycerol, L-arabinose and D-xylose, the specific activities of G6PD and 6PGD are at least fivefold higher than the activities of $NADP^+$-ICD and $NADP^+$-ME (Singh *et al.*, 1988), which indicates that the pentose phosphate pathway is the primary source for NADPH on these carbon sources. Similar conclusions were drawn from an enzymatic analysis in the yeast *Candida utilis* (Bruinenberg *et al.*, 1983b) as well as from radiospirometric experiments in *S. cerevisiae* (Lagunas and Gancedo, 1973). Furthermore the cytoplasmic localisation of the pentose phosphate pathway is consistent with a role in NADPH production. A third argument for the importance of the pentose phosphate pathway in the generation of NADPH in *A. nidulans* is that the flux through the pathway responds to changes in the amount of NADPH consumption. In *A. nidulans* increased demand for NADPH, during growth on nitrate, results in an increase of the amount of G6P metabolised through the pentose phosphate pathway (Carter and Bull, 1969) and increased levels of its enzymes (Hankinson and Cove, 1974). Finally, a fourth argument for the importance of G6PD and 6PGD in the generation of NADPH is the phenotype of the mutants *ppp*A and *ppp*B. These mutants were isolated by their inability to grow on D-xylose, L-arabinose and D-glucuronate and turned out to grow very poorly on nitrate and nitrite as a sole nitrogen source (Hankinson, 1974). *ppp*A mutants were shown to have elevated levels of G6PD, 6PGD, transketolase, glucose-phosphate isomerase and mannitol-6-phosphate dehydrogenase activity, but exhibit a strongly reduced transaldolase activity. By contrast the *ppp*B mutants have decreased activities of all aforementioned enzymes. From these data Hankinson (1974) concluded that *ppp*A most probably encodes transaldolase while *ppp*B probably encodes a regulatory protein of the pentose phosphate pathway. Because both mutations block the flux of metabolites through the pentose phosphate pathway, which probably leads to decreased NADPH production, the reduced growth of these mutants on nitrate, D-xylose and L-arabinose could be the result of a NADPH-shortage. On the other hand, the accumulation of sedoheptulose-7-phosphate in both mutants, could equally well explain the decreased growth on these media.

On carbon sources other than glucose, glycerol, L-arabinose and D-xylose however, the other enzymes might play a more prominent role in the generation of NADPH. For example the regulation of $NADP^+$-ICD (Fig. 1) enzyme activity suggests, that it plays an important role in NADPH production when acetate is used as sole carbon source. $NADP^+$-ICD enzyme activity can be demonstrated in the cytoplasm and the mitochondria of *A. nidulans* mycelium grown on glucose, glycerol, ethanol, D-xylose and L-arabinose, but on acetate the enzyme activity increases fivefold (Singh *et al.*, 1988). The acetate induction of $NADP^+$-ICD enzyme activity is subject to carbon catabolite repression (Kelly and Hynes, 1981b). The $NADP^+$-ICD expression seems to respond to changes in NADPH demand, since on all aforementioned carbon sources, except D-xylose, a higher $NADP^+$-ICD activity was found in mycelia grown on nitrate compared to ammonia (Singh *et al.*, 1988). A further important indication for the role

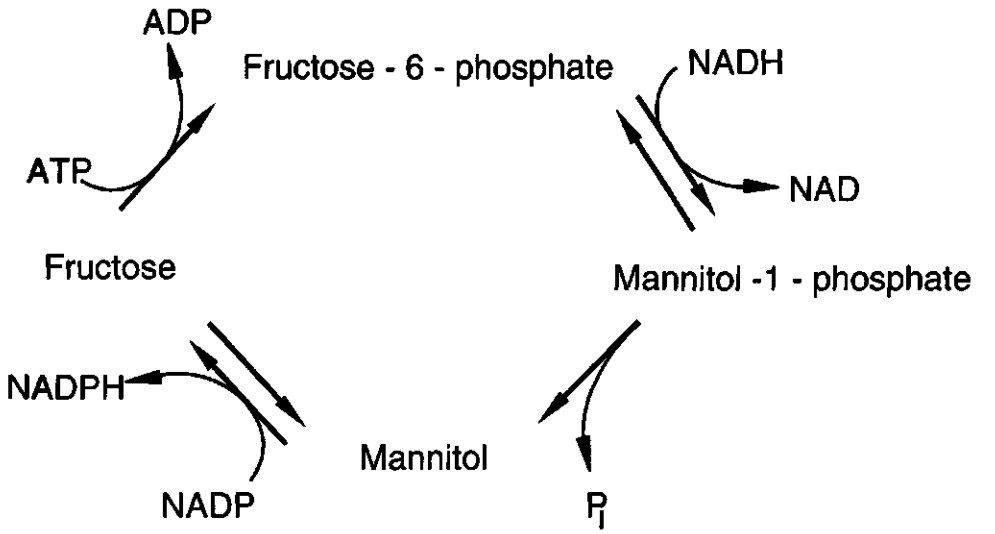

Figure 2. The Mannitol Cycle.

This cyclical pathway, proposed by Hult and Gatenbeck (1978), shuttles hydrogen atoms from NADH to NADP at the expense of ATP. First fructose is phosphorylated (1) by hexokinase (EC 2.7.1.1). The resulting fructose-6-phosphate is reduced to mannitol-1-phosphate with NADH (2) by mannitol-1-phosphate dehydrogenase (EC 1.1.1.17). At its turn, mannitol-1-phosphate is dephosphorylated to mannitol (3) by mannitol-1-phosphate phosphatase (EC 3.1.3.21). Finally, the mannitol is reconverted to fructose (4) via an oxidation with NADP+ catalysed by mannitol dehydrogenase (EC 1.1.1.138).

of NADP$^+$-ICD in NADPH production is that the induction by acetate or nitrate affects primarily the cytosolic isozyme (Singh *et al.*, 1988).

NADP$^+$-ALD (Fig. 1) has been shown to contribute to NADPH generation during growth on ethanol in *C. utilis* (Bruinenberg, 1983a). Studies, in which steady-state *ald*A mRNA levels, encoding NADP$^+$-ALD, were monitored under a number of physiological conditions, suggest that this might also be the case in *A. nidulans* and *A. niger*. In both fungi, steady-state *ald*A mRNA levels are increased during growth on ethanol compared to glucose. Furthermore, the presence of glucose reduces the induction of *ald*A by ethanol in both fungi (O'Connell and Kelly, 1988).

NADP$^+$-ME converts malate to pyruvate (Fig. 1) and is localised in the mitochondrion and the cytosol (Singh *et al.*, 1988). The NADP$^+$-ME enzyme is part of the pathway that shuttles acetyl-CoA from the mitochondrion to the cytoplasm for the biosynthesis of fatty acids (Stryer, 1981). In *A. nidulans* low levels of the enzyme can be demonstrated upon growth on glucose, glycerol, acetate, D-xylose, and L-arabinose. Increased NADP$^+$-ME enzyme activity is found in mycelium grown on the carbon sources ethanol (Singh *et al.*, 1988) and L-proline (Kelly and Hynes, 1981a). The presence of sucrose inhibits this induction and therefore NADP$^+$-ME expression is probably also subject to carbon catabolite repression (Kelly and Hynes, 1981a).

In some organisms transhydrogenases transfer reduction equivalents directly from NADH to NADP$^+$ or vice versa. This reaction can either be energy dependent or energy independent. Such enzymes have been detected in the inner membrane of mammalian mitochondria (Rydström, 1979), but could not be detected in the yeast *C. utilis* (Bruinenberg *et al.*, 1983a). Although the presence of such enzymes in As-

pergilli has not been investigated, the localisation in the inner membrane of mammalian mitochondria make a significant contribution to the cytosolic NADPH pool unlikely (Bruinenberg, 1983a). Similarly a NAD^+ kinase, like the one detected in *S. cerevisiae* mitochondria (Griffiths and Bernofski, 1970), should not contribute significantly to cytosolic NADPH production in Aspergilli.

Indirect shuttling of reduction equivalents from NADH to $NADP^+$ however, has been reported. In the fungus *Alternaria alternata* radiospirometric measurements traced the origin of NADPH used for lipid biosynthesis to a mannitol shuttle (Hult and Gatenbeck, 1978). In this mannitol cycle (Fig. 2) reduction equivalents were shuttled from NADH to $NADP^+$ at the expense of ATP by a cyclical interconversion of fructose and mannitol. The enzymes required for the operation of the mannitol shuttle have been detected in *A. niger* as well as in other *fungi imperfecti* (Hult *et al.*, 1980). However, Singh and coworkers (1988) did not observe a co-ordinated increase of the involved enzymes in *A. nidulans* in response to an increased NADPH demand. These observations demonstrate that the presence of all the relevant enzymes should not be taken as proof for the actual operation of the mannitol-shuttle.

The non-oxidative branch of the pentose phosphate pathway

The end product of the oxidative branch of the pentose phosphate pathway is ribulose-5-phosphate (Ru5P) which in the non-oxidative branch of the pathway via isomerization reactions can be transformed to other pentose phosphates. With the aid of the enzymes transketolase and transaldolase these pentose phosphates can be converted to sedoheptulose-7-phosphate (S7P), fructose-6-phosphate (F6P), erythrose-4-phosphate (E4P) and glyceraldehyde-3-phosphate (G3P). Ribose-5-phosphate (R5P) is a precursor for nucleotide biosynthesis and E4P a precursor for aromatic amino acids. F6P and G3P can enter the glycolytic pathway to be converted to pyruvate or the gluconeogenetic pathway to be reconverted to G6P. The link with the glycolytic and gluconeogenetic pathways endows the pentose phosphate pathway with inherent flexibility, enabling it to produce NADPH, nucleotides and aromatic amino acids in various stoichiometric ratios with respect to G6P. For example, it allows the pentose phosphate pathway to either oxidise G6P completely to CO_2, generating the maximum amount of 12 NADPH or to convert it to R5P generating only 2 NADPH. All reactions in the non-oxidative branch of the pentose phosphate pathway are completely reversible (Bonsignore *et al.*, 1962). Therefore all precursors for biosynthesis of aromatic amino acids or nucleic acids can be derived from glycolytic intermediates by reversal of the transaldolase and transketolase catalysed reactions (Bonsignore *et al.*, 1962). This is probably the reason that G6PD mutants are still able to synthesise these compounds.

Glucose-6-phosphate dehydrogenases

Glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) catalyses the conversion of glucose-6-phosphate into 6-phosphogluconate, which is the first step of the pentose phosphate pathway (Levy, 1979). Together with 6-phosphogluconate dehydrogenase (6PGD; 6-phospho-D-gluconate:$NADP^+$ 2-oxidoreductase, EC 1.1.1.44), G6PD makes up the oxidative branch of the pentose phosphate pathway. NMR experiments demonstrated that the dehydrogenation of glucose-6-phosphate (G6P) can proceed via two lactone intermediates; either 6-phosphoglucono-δ-lactone or 6-phosphoglucono-γ-lactone (Jarori and Maitra, 1991). The inherent instability of especially the δ-lactone reaction product, makes the G6PD reaction virtually irreversible under physiological conditions (Beutler and Kuhl, 1985b, Beutler and Kuhl, 1986). Additionally, in human and rat erythrocytes

there is a 6-phospholactonase, which ensures the efficient removal of δ- and γ-lactone (Beutler and Kuhl, 1985b). Therefore, under physiological conditions, the G6PD reaction can be regarded as the committing step in the oxidative branch of the pentose phosphate pathway.

In traditional biochemistry, flux regulation in a metabolic pathway is often assumed to take place on a rate limiting step. G6PD is often described as the main regulatory enzyme, that determines the flux through the pentose phosphate pathway (Ho *et al.*, 1988, Fouts *et al.* 1988). However, there are no experimental data, that support the rate limiting character of the reaction catalysed by G6PD. In contemporary biochemistry the concept of metabolic flux control by regulation at a rate limiting step is replaced by the theory of metabolic control analysis (MCA). In this new theory, control over a metabolic flux is distributed over all the enzymes of a metabolic pathway and more than one enzyme can exert significant control over the flux through a metabolic pathway. Additionally, the distribution of control can change in a metabolic state or by external conditions (Quant, 1993). Experimental support for this theory was, amongst others, obtained with the tryptophan biosynthetic pathway in *S. cerevisiae*. In the tryptophan biosynthetic pathway, manipulation of the activity of the individual enzymes, over a 10- to 50-fold range, did not have great impact on the flux through the pathway. Only a co-ordinated 20-fold increase of all the enzyme activities involved, resulted in a 9-fold increase in tryptophan biosynthesis (Niederberger *et al.*, 1992). The pentose phosphate pathway could also be subject to this kind of regulation. For example in *A. nidulans* an increased flux of metabolites through the pentose phosphate pathway is not only accompanied by an increase in the G6PD activity, but also by a similar increase of all the other enzyme activities in the pathway (Carter and Bull, 1969; Hankinson and Cove, 1974).

Since its discovery in horse erythrocytes by Warburg and Cristiansen (1931), an impressive body of biochemical data on G6PD enzymes, isolated from a great variety of sources, has been accumulated. G6PD is an ubiquitous enzyme, for it has been detected in all organisms tested (Levy, 1979). G6PD enzymes differ considerably in their cofactor specificity. Levy (1979) distinguished five classes of G6PDs according to their pyridine dinucleotide specificity: enzymes which are completely specific to either NAD^+ or $NADP^+$, enzymes which prefer either cofactor, but show some enzyme activity with the other, and enzymes that have no preference whatsoever. All eukaryotic G6PDs are either $NADP^+$-specific or $NADP^+$-preferring, which fits their role as NADPH producers.

The NAD^+-specific, NAD^+-preferring or dual specificity G6PDs occur only in prokaryotes, where G6PD takes part in other metabolic routes than the pentose phosphate pathway. The best characterised example of a G6PD enzyme with a dual dinucleotide cofactor specificity is the one from the lactic acid bacterium *Leuconostoc mesenteroides* (Lee *et al.*, 1991). Glucose degradation by this G6PD generates either NADPH for lipid biosynthesis or NADH for the synthesis of lactic acid via the Entener-Doudoroff pathway (Levy, 1979). The sole example of a truly NAD^+-specific G6PD is found in *Acetobacter xylinium*, where it exists alongside a $NADP^+$-specific G6PD. These two G6PDs are encoded by separate genes (Levy and Cook, 1991)

Apart from their cofactor specificity, G6PD enzymes from different organisms show considerable variation in substrate specificity, kinetic parameters, mechanism of catalysis and allosteric modulators. And although a detailed discussion of the biochemical characteristics of the G6PDs from different sources is beyond the scope of this introduction (for review see Bonsignore and De Flora, 1972; Levy, 1979), these data provide important indications for the complex regulation of G6PD, especially in eukaryotic cells. For example these data show that G6PD enzyme activity can be modulated through interactions with allosteric effectors. The most common inhibitor is NADPH, but some G6PDs are inhibited by ATP and/or long chain fatty acid thioesters and mammalian G6PDs are specifically inhibited by steroid hormones (Levy, 1979).

The active form of the enzyme is reported to be either a dimer or a tetramer, but there are conflict-

ing reports on whether these G6PD multimers are assembled from identical or different monomers (for a review see Levy, 1979).

The advent of recombinant DNA techniques and the subsequent cloning of a number of pro- and eukaryotic G6PD encoding genes opened the possibility to study the regulation of G6PD at the molecular level. Isolation and characterisation of G6PD mutants in humans, *S. cerevisiae*, *D. melanogaster*, *E. coli* and *Neurospora crassa* provided further insight into the physiological role of G6PD, the regulation of G6PD expression and protein structure-function relationships. To illustrate this, I would like to discuss briefly the information on the regulation of G6PD expression and the phenotypes of G6PD mutants in the aforementioned species.

Cloned G6PD encoding genes

The human G6PD encoding gene Gd

The discovery that G6PD deficiency is the cause of drug-induced haemolytic anaemia in man aroused the attention of the scientific community in the early fifties. As a direct consequence of this discovery, to date approximately 380 variants of human G6PD have been identified biochemically (Beutler, 1989, 1991). G6PD deficiency, as result of mutations in the G6PD encoding gene (*Gd*), is the most common form of genetic disorder in man. Recent studies estimate that 200 million people are affected (Beutler, 1991). However, some G6PD variants are not associated with reduced enzyme activity and therefore have no clinical consequences (Beutler, 1989). On the other hand, patients, carrying other G6PD variants, suffer either from chronic or periodic haemolytic anaemia depending on the residual G6PD activity (Beutler 1991; WHO working group, 1989). This anaemia is a result of depletion of reduced glutathione in erythrocytes due to low NADPH production by an abnormal G6PD enzyme, which in turn leads to oxidation of sulfhydryl groups in proteins. As a consequence, the membranes of erythrocytes from patients with chronic hemolysis contain complexes of spectrin and other proteins covalently bonded by intermolecular disulphide bridges (Johnson *et al.*, 1985). Similar phenomena have been observed in glucose-starved normal erythrocytes and thus occurrence of these protein complexes seems to be directly linked to low G6PD activity (Schulman *et al.*, 1980). How these protein complexes are related to the observed hemolysis is still poorly understood, but the increased rigidity of G6PD-deficient erythrocytes seems to play a role (Johnson *et al.*, 1985).

Although G6PD is expressed in all tissues, the clinical manifestations of G6PD deficiency are almost completely limited to erythrocytes. Only in rare cases, other cell types are also affected by the G6PD deficiency (Beutler, 1991). For example in polymorphonuclear leukocytes, inability to assemble microtubuli has been reported as a result of oxidation damage caused by lack of reduced glutathione. This defect can be reversed by addition of vitamin B, which causes the leukocytes to produce less hydrogen peroxide (Schulman *et al.*, 1980).

These observations suggest that especially cells exposed to oxidation stress are sensitive to decreased levels of G6PD enzyme activity. Experiments with rabbit lung tissue have shown that specific inhibition of G6PD enzyme activity leads to increased sensitivity to superoxide generating compounds (Heffner and Milam, 1990). Moreover, disruption of the G6PD gene in an embryonic mouse cell line (Pandolfi *et al.*, 1995), rendered these cells extremely sensitive to oxidative stress. Apart from their sensitivity to oxidative stress, these G6PD null-mutants did not appear to have any other defects.

Still, because of the involvement of NADPH in a large number of biosynthetic reactions, it remains peculiar that almost no adverse effects of G6PD deficiency seem to occur in other cell types than erythrocytes. To resolve this paradox, one could assume that most cells require less NADPH than erythro-

cytes do. The latter would imply that NADPH consumption and thus the level of G6PD enzyme activity should vary among tissues. In fact, considerable variation in G6PD activity amongst different tissues has been reported for human liver; G6PD enzyme activity is low in the perivenous area and high in the periportal region (Wimmer *et al.*, 1990).

A second explanation would be that other enzymes take over the NADPH production in G6PD-deficient cells. For example, Beutler (1991) suggested that the presence of hexose-6-phosphate dehydrogenase (ß-D-glucose:$NADP^+$ 1-oxidoreductase, EC 1.1.1.47) would alleviate the consequences of G6PD deficiency. However, the localisation of hexose-6-phosphate dehydrogenase within the cisternae of the endoplasmatic reticulum (ER) together with all other enzymes of the pentose phosphate pathway (Bublitz and Stevenson, 1988), suggest the existence of a separate pentose phosphate pathway in the ER. If these enzymes act as an ER-specific pentose phosphate pathway, it is unlikely that hexose-6-phosphate dehydrogenase activity contributes to the cytosolic NADPH pool. Additionally, the capacity of the microsomal pentose phosphate pathway to produce NADPH is limited; it has only about 5% of the activity of its cytoplasmic counterpart (Bublitz and Stevenson, 1988). The question whether and to what extent other NADPH producing reactions can compensate for the effects of G6PD deficiency in man has still not been properly addressed.

The observation that all characterised G6PD variants in man retain enzyme activity, stresses the importance of G6PD. All but one G6PD variant are the result of a point mutation and the only known deletion mutant has a three base pair deletion. The latter suggests that a human G6PD-null mutant is not viable (Vulliamy *et al.*, 1992). The high incidence of G6PD deficiency has been explained by the protection against malaria conferred by this mutation. The mechanism of this protection is unclear, but it is suggested that the increased oxidative stress in G6PD-deficient erythrocytes inhibits the growth of the parasite (Roth and Schulman, 1989).

Isolation and sequence analysis of cDNA clones encoding the human G6PD (Persico *et al.*, 1986), allowed the G6PD variants to be characterised at the DNA level. DNA analysis proved to be a more dependable and, since the introduction of DNA amplification by the polymerase chain reaction (PCR), a faster method than biochemical characterisation of the mutant enzyme (Beutler, 1989).

The genomic sequence of the human G6PD encoding gene (*Gd*) was found to contain promoter elements typical for a mammalian housekeeping gene: a TATA-box-like element, several GGGCGG hexanucleotides representing putative binding sites for the mammalian transcription factor SP-1, a sequence homologous to the SV40 21 bp repeat and a CpG-rich region (Martini *et al.*,1986; Chen *et al.*, 1991). The CpG-rich regions in the *Gd* promoter probably play a role in the phenomenon of X-chromosome inactivation in females. The human *Gd* gene is located on the X-chromosome and consequently females have two copies of the *Gd* gene. To compensate for the two copies of each of the X-linked genes in each cell, one of the X-chromosomes is inactivated. Analysis of DNA from males and females showed hypomethylation at specific sites within the CpG-rich region or CpG islands of active X-chromosomes compared to inactive chromosomes (Toniolo *et al.*, 1990).

To study the functional organisation of the *Gd* gene, cultured cells were transiently cotransfected with plasmids containing the human *Gd* promoter fused to the chloramphenicol acetyl transferase (CAT) reporter gene. A mere 436 bp upstream of the transcription start proved to be sufficient to drive high expression of the CAT gene. Site directed mutagenesis of the TATA-box-like sequence resulted in altered transcription starts but not in lower CAT expression (Ursini *et al.*, 1990). The level of CAT activity was unexpected, since Northern blot analysis showed low steady state levels of *Gd* mRNA, comprising only 0.02% of the $polyA^+$ mRNA in human fibroblasts (Toniolo *et al.*, 1984). This observation prompted the authors to speculate that human *Gd* expression is not regulated at the transcription level. The data presented by Ursini and co-workers (1990), do not exclude the possibility that the high level

of CAT transcription could also be an artefact of transient expression. Nevertheless, there are reports that post-transcriptional regulation of G6PD expression occurs in human erythrocytes (Kahn, 1976), rat liver and rat erythrocytes (Nehal *et al.*, 1990).

The Rat G6PD gene

From rat, only the G6PD cDNA sequence has been published (Ho *et al.*, 1988). Still, interesting data concerning the regulation of G6PD expression at transcription level have been obtained in physiological studies.

In rats a regime of fasting, followed by refeeding with a carbohydrate-rich diet or administration of insulin induce an increase of G6PD, 6PGD and $NADP^+$-linked malic enzyme activity in liver (Tepperman and Tepperman, 1964). The elevated steady state levels of G6PD mRNA (Kletzien, 1986) indicate, that insulin and the fasting/refeeding regime influence the transcription level and/or the stability of G6PD mRNA.

Both insulin and fasting/refeeding stimulate lipid biosynthesis in rat liver (Miksisek and Towle, 1982), which suggests that G6PD enzyme activity is induced to provide the required NADPH. The latter is supported by the observation that G6PD activity does not increase upon refeeding with fat (Tepperman and Tepperman, 1964). Moreover, insulin administration does not increase G6PD and 6PGD enzyme activity, when the NADPH consuming reactions are blocked by inhibitors (Ayala *et al.*, 1990). The latter suggests that NADPH consumption is the elicitor of the G6PD and 6PGD induction. However, the insulin dependent increase in G6PD mRNA is inhibited by cycloheximide, which suggests that not only NADPH consumption but also protein synthesis is required (Katsurada *et al.*, 1989).

Monitoring rat liver G6PD activity, steady state G6PD mRNA levels and the G6PD transcription rate, Katsurada (1989) found that in diabetic rats G6PD mRNA levels are markedly decreased as compared to those in normal rats. Insulin administration completely restores G6PD mRNA levels and G6PD enzyme activity in diabetic rats. A shift from a glucose-rich to a fructose-rich diet restores the steady state level of G6PD mRNA in diabetic rats to only 50% of the level in normal rats. By contrast, the G6PD enzyme activity is not restored to the same extent as the mRNA level by such a shift to a fructose-rich diet. Therefore, Katsurada and co-workers (1989) suggest that insulin not only stimulates transcription of the G6PD gene, but is also involved in the translation of the G6PD mRNA.

Another factor that appears to influence the G6PD enzyme levels in rats is the regulation of G6PD mRNA stability. In response to a fasting and refeeding dietary regime, female rats exhibit a lower induction of G6PD mRNA and G6PD enzyme activity than males. Since the transcription activity is identical in both sexes, this difference has been attributed to a difference in G6PD mRNA stability between the genders (Holten *et al.*, 1993).

The *Drosophila melanogaster Zw* gene, encoding G6PD

The G6PD encoding gene *Zw* from *D. melanogaster* has been cloned (Hori *et al.*, 1985) and sequenced (Fouts *et al.*, 1988), but no expression studies have been performed. The abbreviations *Zw*, for the *Drosophila* G6PD encoding gene, and *zwf* for the *S. cerevisiae* and *E. coli* genes are derived from the German "Zwischenferment", the name originally given to the G6PD enzyme by its discoverers Warburg and Cristiansen (1931).

G6PD null mutants of *D. melanogaster* were isolated as suppressor mutations of the lethal 6PGD

mutations. Mutation of the G6PD encoding gene *Zw* prevents the accumulation of 6-phosphogluconate and the lethal phenotype of the 6PGD mutation is suppressed (Gvozdev *et al.*, 1977). The *Pgd/Zw* double mutants do not have an appreciable phenotype, suggesting that the oxidative part of the pentose phosphate pathway is not essential in *D. melanogaster*.

Although there are no instantaneous consequences of G6PD mutations in *D. melanogaster*, recent studies suggest that there might be long term effects. Like in humans, G6PD variants have been discovered in natural populations of *D. melanogaster*: Zw^a, Zw^b and Zw^{lo1}. These variants represent a tetrameric, dimeric and a monomeric form of the enzyme respectively (Williamson and Bentley, 1983). The dimeric form (Zw^b) exhibits 50% of the activity of the tetramer (Zw^a), whereas the monomeric form (Zw^{lo1}) retains only 2% of the activity of Zw^a. In populations selected for long or short life span, the Zw^a is invariably associated with longevity and the low activity variant Zw^b with short life span (Luckinbill *et al.*, 1990). It would therefore be interesting to see, whether G6PD mutations that decrease G6PD enzyme activity also reduce the human life span.

ZWF1, the G6PD-encoding gene from *S. cerevisiae*

Radiospirometric measurements showed that the pentose phosphate pathway in glucose grown *S. cerevisiae* is the primary source for NADPH (Gancedo and Lagunas, 1973). A G6PD- deficient mutant of *S. cerevisiae* was isolated as a pseudo-revertant of 6PGD (*gnd*) mutations on glucose containing medium. After segregation from the *gnd* mutation, the *zwf1* mutation conferred no phenotype to *S. cerevisiae* (Lobo and Maitra, 1982).

When the cloned *ZWF1* gene was used to construct a *zwf* null mutation by gene disruption, it proved to have no other phenotype than a slightly increased sensitivity towards superoxide generating compounds (Nogae and Johnstone, 1990). Paradoxically, the *ZWF1* gene from *S. cerevisiae* was recently cloned by selection for complementation of *met*19, a mutation causing a methionine requirement (Thomas *et al.*, 1991). In this case, disruption of the *ZWF1* gene resulted in a (null) mutant, that exhibited an absolute requirement for an organic sulphur source like methionine, cysteine, homocysteine, glutathione or S-adenosyl methionine. A shortage of NADPH to reduce sulphate, as a direct consequence of G6PD deficiency, could explain this phenotype. However, no growth was observed on reduced sulphur compounds like sulphides, which rules out a lack of NADPH as cause for the organic sulphur requirement. Furthermore, only poor growth of the null-mutant was observed on cysteine, ruling out in-ability to synthesise glutathione. In wild type *S. cerevisiae*, addition of methionine to the growth medium decreased *ZWF1* transcription with 50%. No satisfactory explanation for this phenomenon has been found yet. The organic sulphur requirement was missed by Nogae and Johnstone (1990) because their parental strain already carried a *met* mutation. Also Lobo and Maitra (1982) do not mention any organic sulphur requirement for their *zwf1* mutant, which could be caused by the low residual G6PD activity of this mutant. On the other hand Lobo and Maitra state neither the composition of their media nor growth conditions, so the *met* phenotype of the *zwf1* mutant might simply have escaped their attention.

The *Escherichia coli zwf* gene

Like in *D. melanogaster* and *S. cerevisiae*, *Escherichia coli* G6PD (*zwf*) mutants were isolated as pseudo revertants of mutations in the 6PGD encoding *gnd* gene (Fraenkel, 1968: Fraenkel and Vinopal,

1973). The resulting *zwf/gnd* double mutants have no apparent phenotype which indicates that the pentose phosphate pathway is not essential for NADPH production in *E. coli*. Similarly, *zwf/icd* double mutants grow on glucose as sole carbon source, which indicates that $NADP^+$-linked isocitrate dehydrogenase (*icd*) is not essential for NADPH generation either.

Radiospirometric studies, conducted in wild type, *zwf* and *icd* mutants, confirmed that neither the dehydrogenases of the pentose phosphate pathway nor $NADP^+$-ICD are major contributors to NADPH production. These studies nevertheless demonstrate that in *E. coli* hydrogen atoms from G6P are transferred somehow to $NADP^+$. The pathway responsible for this hydrogen transfer could not be identified (Csonka and Fraenkel, 1977).

Paradoxically, data from other studies in *E. coli* do indicate that G6PD plays a role in NADPH production. In *E. coli* G6PD and 6PGD enzyme activity was shown to increase with increasing growth rate (Wolf *et al.*, 1979). For G6PD, the growth rate dependent level of enzyme activity is regulated at the level of *zwf* transcription (Rowely *et al.*, 1991). In the case of 6PGD this growth rate dependent regulation occurs at the level of translation initiation (Carter-Muenchau and Wolf, 1989). The growth rate dependent regulation of the two dehydrogenases of the pentose phosphate pathway could be the result of an increased demand for pentoses as precursors for nucleotide synthesis rather than a response to an increased demand for NADPH. However, in *E. coli* G6PD is also induced by the products of the regulatory genes *soxR/soxS*, which mediate the superoxide stress response (Greenberg and Demple, 1992). Under these conditions G6PD most probably acts as a producer of reduction equivalents for the detoxification of superoxides and peroxides.

G6PD deficient mutants in *Neurospora crassa*

In contrast with the previous examples, mutations leading to G6PD deficiency in the filamentous fungus *Neurospora crassa* seem to have a profound effect on NADPH production. In *N. crassa*, the four unlinked mutations *col, bal, fr* and *ace* give rise to G6PD enzymes with altered catalytic and electrophoretic properties. The mutants *col, bal* and *fr* were isolated because of their grossly altered colony morphology and strongly reduced growth rates (Scott and Tatum, 1970). The *ace*-7 mutant was isolated because of its acetate requirement. In the *ace*-7 mutant, $NADP^+$-ICD and $NADP^+$-ME were strongly induced, probably to compensate for the loss NADPH production by the pentose phosphate pathway (Nishikawa and Kuwana, 1985). All four mutations lead to decreased intracellular NADPH levels. The phenotype of these mutants seemed to be directly linked to the extent of G6PD deficiency; the lower the residual G6PD activity, the lower the intracellular NADPH concentration and the more severe the morphological abnormality (Scott and Mahony, 1974: Nishikawa and Kuwana, 1985).

The biochemical and genetical data have been interpreted by Scott and Mahony (1974) and also by Nishikawa and Kuwana (1985) as proof that *col, bal, fr* and *ace*-7 represent individual G6PD encoding genes in *N. crassa*. A problem with this interpretation is, that one has to explain why a mutation in any of these four G6PD encoding genes, should lead to G6PD deficiency. According to Scott and Mahony (1974), dimers of the different G6PD polypeptides, encoded by the four genes, would associate in different combinations to build the tetrameric G6PD holo-enzyme. As a consequence, a mutation in any of these putative G6PD encoding genes would result in G6PD deficiency. However, conclusive evidence that these four genes represent four G6PD encoding genes in *N. crassa* was never obtained. In fact, Scott and Mahony's (1974) data allow also a completely different interpretation. Instead of four structural G6PD genes, only one of the *ace*-7, *col, bal* or *fr* genes could represent a G6PD encoding gene. The remaining three genes could play a role in the modification of this single G6PD polypeptide.

The recent finding that the G6PD in *S. cerevisiae* is a glycoprotein (Reilly and Allfred, 1995) demonstrates clearly that modification of G6PD occurs.

The data on the different G6PD enzymes, genes and mutants, summarised so far, show that there is some consensus but that there are also large differences. Even contradictory results have been obtained for *E. coli* and *S. cerevisiae* in different studies. The phenotypes of the G6PD mutants, especially the phenotype of *S. cerevisiae zwf* null-mutants, indicate that the textbook statement that G6PD is the primary supplier of NADPH (Stryer, 1981), does not have universal validity. Therefore, to asses the physiological significance of G6PD better, more research is necessary.

The already existing knowledge on its carbon metabolism, its metabolic versatility and the availability of well characterised mutants, make *A. nidulans* the model system of choice for this research. Additionally, the work of Hankinson and Cove (1974) suggested already a relation between the regulation pentose phosphate pathway and NADPH consumption in *A. nidulans*. The carbon metabolism in *A. niger* is less characterised, but its use in industrial fermentations requires insight in the regulation of its metabolism. An approach would be to clone the G6PD encoding genes from *A. nidulans* and *A. niger* and to study G6PD expression under various physiological conditions. The physiological conditions of choice would be those that are known to influence NADPH consumption. Since the expression studies in rat, *S. cerevisiae* and *E. coli* clearly indicate, that at least part of the G6PD response to different stimuli is regulated at the transcription level, we chose to start our study on that level. If G6PD steady state mRNA levels respond to a metabolic stimulus, the G6PD encoding gene could contain the relevant responsive *cis*-acting elements. A next step, could then be to attempt to locate these *cis*-acting elements by a promoter deletion study.

Additionally, this approach has the advantage that it also yields information on fungal promoters. Only few promoters from filamentous fungi have been characterised and little is known about the *cis*-acting elements they contain. Most of our knowledge on the regulation of transcription by RNA polymerase II in eukaryotes has been deduced from studies on promoters from higher eukaryotes and the ascomycete *S. cerevisiae*. Therefore, I would like to discus briefly the *cis*-acting elements in mammalian and *S. cerevisiae* RNA-polymerase II promoters before discussing strategies to detect them in promoters from filamentous fungi.

Eukaryotic RNA polymerase II promoters

Recent models of the transcription regulation in eukaryotes state, that regulatory regions of DNA responsible for directing transcription contain multiple recognition
elements that interact with sequence-specific DNA-binding proteins. Some of these DNA-binding proteins assist in the co-ordinated assembly of the transcription initiation complex (for a review see Dynan and Tjian, 1985: Ptashne, 1988).

Sequence comparison of promoters from mammalian genes revealed that they can be divided into three groups. The first group (Fig. 3) contains a sequence with the consensus TATA approximately 25 to 30 bp upstream of the transcription start point (tsp). The second group (Fig. 3) lacks obvious TATA-boxes, some of which are GC-rich and are primarily encountered in housekeeping genes. Such promoters have several binding sites for the transcription initiation factor SP-1 and several transcription start points (tsps), spread over a fairly large region (Sehgal *et al.*, 1988). A third group has neither a TATA-box nor contains GC-rich sequences. The latter type of promoters have either one or two tightly clustered tsps (Smale and Baltimore, 1989). For several mammalian cellular and viral promoters it has been

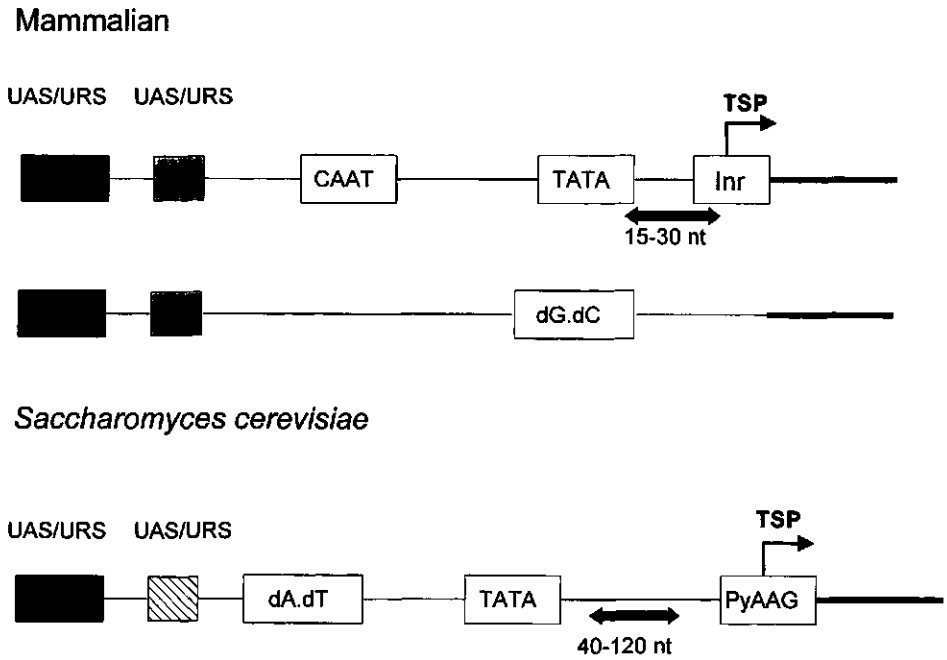

Figure 3. *Cis*-acting elements in mammalian and *S. cerevisiae* RNA-polymerase II promoters.
The *cis*-acting elements in mammalian and *S. cerevisiae* promoters can be divided in three groups: 1) TATA-box (**TATA**), initiator (**Inr**) and the **PyAG** motif that make up a minimal or core promoter that is able to drive a basal level of transcription, 2) CCAAT box (**CCAAT**), AT- (**dAdT**) and GC-rich (**dGdC**) sequences which increase the basal transcription level in a large number of genes and are therefore regarded as general transcription activating sequences. 3) upstream activating (**UAS**) or repressing (**URS**) sequences that modulate the transcription of a limited number of genes. The TATA-box plays a central role in transcription initiation. The transcription factor IID (TFIID) that binds to TATA-box, initiates the ordered assembly of other general transcription factors (IIA, IIB, IIE and IIF) and RNA polymerase II into a large multiprotein initiation complex ((Sawadogo and Roeder, 1985, van Dyke *et al.*, 1988, Buratowski *et al.*, 1989: Conaway and Conaway, 1990, Lin *et al.*, 1991, Dynlacht *et al.*, 1991). Several observations indicate that this holo-TFIID complex plays a central role in the transcription of mammalian and *S. cerevisiae* genes. First, the holo-TFIID complex not only supports transcriptional activation of TATA-box containing promoters by diverse activators but also of TATA-less promoters (Zhou *et al.*, 1992). Second, TBPs have also been shown to be necessary for transcription initiation by RNA polymerase I and for transcription by RNA polymerase III (Comai *et al.*, 1992). Third, the yeast and human TFIID's are functionally interchangeable for the response to acidic transcriptional activators *in vitro* (Kellerher III *et al.*, 1992). It could therefore very well be that TBP is a truly universal eukaryotic transcription factor.

The basal level transcription is modulated by interaction of the transcription initiation complex with general transcription factors or with more specific transcription factors binding to UAS's or URS's (for a review see Dynan and Tjian, 1985: Ptashne, 1988). The linear order of the transcription modulating elements with respect to TATA-box, is the major determinant of the direction of transcription (Xu *et al.*, 1991). How the interaction between the transcription initiation complex and the transcription factors changes the level of transcription is unknown. The interaction does not seem to increase the affinity of the transcription initiation complex for its DNA-binding sites. For example in the case of the GC-box-binding transcription factor SP-1, it has been demonstrated that it stimulates transcription *in vitro* without enhancing DNA-binding activity of the TATA-box factor. Instead, SP-1 appears to act by increasing the number of productive transcription complexes (Schmidt *et al.*, 1989).

established that a TATA-box and a transcription initiation site, or even only a TATA-box, can form a minimal or core promoter, which is able to drive a basal level of transcription.

Several types of *cis*-acting sequence elements can be involved in the modulation of the basal level of transcription. Some of these transcription modulating sequences like CCAAT- or GGGCGG homologies are found in many different mammalian promoters and therefore can be regarded as general mammalian transcription activating sequences. Other transcription modulating sequences like the metallothionin metal regulatory element (Karin, 1984) or the heat-shock regulatory element (Pelham, 1982) are encountered in only a limited number of promoters and hence seem to have a more specialised role (Dynan and Tjian, 1985). In different groups of organisms, different sequence motifs seem to act as general transcription activating elements. For example, many promoters in *S. cerevisiae* contain poly dA-dT sequences (Fig. 3), that appear to be important for constitutive expression (Struhl, 1985). CCAAT-boxes, however are rarely found in promoters of *S. cerevisiae* (Nussinov, 1990).

Both the CCAAT- and GGGCGG (GC)-boxes are located immediately upstream of the tsps (Fig. 3). The metallothionin metal regulatory element and the heat-shock regulatory element belong to another class of sequence motifs, that are usually located farther upstream of the tsps than the CCAAT- and GC-boxes. Therefore, such elements are referred to as upstream activating (UAS) or upstream repressing sequences (URS). Recently, transcription modulating sequences have also been detected downstream of the tsp, within the coding region of a number of developmental regulatory genes in *D. melanogaster* (Thummel, 1989).

Mutations in mammalian TATA-like sequences resulted in decreased transcription initiation and/or heterogenous tsps (Wasylyk *et al.*, 1980). In other cases however, accurate transcription initiation was observed following specific mutagenesis of the TATA-box (Hen *et al.*, 1982: Jones *et al.*, 1988). Also in many *S. cerevisiae* promoters TATA-box-like sequences can be found, albeit their distance to the tsp is 40-120 bp (Struhl, 1985) instead of the 25 to 30 bp observed for mammalian RNA polymerase-II promoters (Breathnach and Chambon, 1981). The actual site of transcription initiation in *S. cerevisiae* is not determined by the TATA-box, but by specific sequences like PyAAG (Chen and Struhl, 1985).

For several mammalian promoters, the sequences immediately surrounding the tsp have also been implicated in the regulation of transcription initiation. In mammalian promoters that contain a TATA-box, transcription often starts at an A, which is part of a weak consensus sequence PyPyCAPyPyPyPyPy (Corden *et al.*, 1980). A sequence motif in the promoter of the murine lymphocyte-specific terminal deoxynucleotidyltransferase (TdT) gene, that contains the tsp and fits this PyPyCAPyPyPyPyPy consensus, was shown to be sufficient for basal level transcription. This sequence motif from the TdT promoter was named initiator (Inr) (Smale and Baltimore, 1989). The basal level of TdT transcription, driven by the Inr element, can be stimulated by a suitably positioned TATA- or GC-box. The observation that a protein specifically binds to the Inr-like element in the long terminal repeat of a human endogenous provirus (ERV9) (La Mantia *et al.*, 1992), suggests that the Inr is in fact a distinct mammalian promoter element.

General promoter elements in filamentous fungi

Initial attempts to clone genes from filamentous fungi by the complementation of homologous *S. cerevisiae* mutants were largely unsuccessful, suggesting that in general the transcription initiation and/or splicing signals from filamentous fungi are not efficiently recognised in *S. cerevisiae* (Rambosek and Leach, 1987). Still, several authors insist on pointing out the presence of TATA- and CCAAT-box-like motifs in the 5' proximal regions of genes from filamentous fungi (Rambosek and Leach, 1987: Gurr *et*

al., 1987: Unkles, 1992). TATA-box-like motifs occur in many promoters from filamentous fungi at 40 to 100 bp from the tsp (Unkles, 1992). Sequences resembling the CCAAT-boxes in mammalian promoters can be found in 75% of the genes from filamentous fungi (Rambosek and Leach, 1987: Unkles, 1992). Their location is usually 100 to 200 bp upstream of the tsp, but sometimes they are found more than 300 bp away (Unkles, 1992). This means that the distance to the tsp of the CCAAT- and TATA-box-like sequences varies from gene to gene and that the distance of these sequence motifs to the tsp is much larger than in mammalian and *S. cerevisiae* promoters. In fact these distances to the tsp are so large, that the chances of a random occurrence of CCAAT- and TATA-like sequence motifs become considerable.

Deletion of fragments, containing a TATA motif, in the *A. nidulans gpd*A (Punt *et al.*, 1990) and *aba*A (Adams and Timberlake, 1990) promoters did not result in heterogeneous tsps, suggesting that in those genes a TATA motif does not determine the initiation site. Since the TATA motif in the *aba*A and *gpd*A promoters was not specifically deleted or mutated, its effect on transcription efficiency could not be deduced. Specific deletion of the TATA-box in the *A. nidulans oli*C promoter, abolished the major tsp but did not have a significant effect on the transcription level (Turner *et al.*, 1989).

By contrast, proteins that bind specifically to CCAAT motifs have been detected in several *A. nidulans* promoters (van Heeswijk and Hynes, 1991: Ridchardson *et al.*, 1992: Litzka *et al.*, 1996). Moreover, the CCAAT-box sequence in *amd*S is required for setting the basal level of expression (van Heeswijk and Hynes, 1991: Littlejohn and Hynes, 1992). The target sites of the CCAAT-binding proteins in *amd*S, *lam*A, *lam*B and *gat*A are located next to, or overlapping the AMDR-binding site which regulates Ω-amino acid induction these genes (Hynes *et al.*, 1989). The *A. nidulans hap*C gene, that encodes at least a subunit of the CCAAT-binding factor, acting on *amd*S and *gat*A, exhibits extensive homology to the *S. cerevisiae hap*3 gene (Pappagianopoulos *et al.*, 1996). The HAP3 protein, together with the *hap*2, 4 and 5 gene products, makes up the *S. cerevisiae* CCAAT-binding protein complex. The observation that HAP2 and 3 are functionally interchangeable *in vitro* with subunits of the human (Chodosh *et al.*, 1988) and rat (Sinha *et al.*, 1995) CCAAT-binding complex, suggests evolutionary conservation of major parts of the transcription apparatus among eukaryotes. The homology between *hap*C and *hap*3 indicates that *A. nidulans* promoters are no exception in this respect.

C+T-rich sequences are a third sequence motif that is often encountered in promoters of filamentous fungi (Gurr *et al.*, 1987: Unkles, 1992). In the *A. nidulans gpd*A (Punt *et al.*, 1990) and *oli*C (Turner *et al.*, 1989) promoters deletion of the C+T-rich sequences results in aberrant tsps, without significant effect on the overall promoter activity. Both in the case of *gpd*A and *oli*C the aberrant tsps map again downstream a shorter CT-box, suggesting that a CT-box is sufficient for determining the tsp (Punt *et al.*, 1990). It was proposed that transcription initiates at the first purine downstream of a CT-box (Punt and van den Hondel, 1992). However, this may not be true in all cases since in several genes C+T-rich sequences have been observed downstream of the tsp (Unkles, 1992). Pyrimidine-rich stretches or CT-boxes have been described in the promoters of both in *S. cerevisiae* (Struhl, 1985) and filamentous fungi (Gurr *et al.*, 1987: Unkles, 1992). Recently C+T-rich promoter elements were identified in mammalian genes because of their hypersensitivity to both double- and single strand specific nucleases. This nuclease hypersensitivity has been attributed to the fact that these C+T-rich sequences can assume alternate DNA conformations. Furthermore a cDNA was isolated that encoded a human protein (NSEP-1) that binds specifically to CT-rich double stranded DNA as well as to CT-rich single stranded DNA (Kolluri *et al.*, 1991).

A core promoter in filamentous fungi?

The data from the *A. nidulans trp*C (Hamer and Timberlake, 1987), *aba*A (Adams and Timberlake (1990), *oli*C (Turner *et al.*, 1989) and *gpd*A (Punt *et al.*, 1990) promoter deletion studies, support the existence of a core or minimal promoter in genes from filamentous fungi. Within this putative filamentous fungal core promoter, separate elements seem to govern the basal level of transcription and the position of transcription initiation. However, which sequence motifs in the core promoter are responsible for the regulation of basal level transcription and correct transcription initiation is still unclear. Because of the limited number of promoter analyses performed in filamentous fungi, no general statement can be made on the functionality of the sequence motifs that resemble mammalian CCAAT- and TATA-boxes. Sequences that resemble the mammalian initiator (Inr) element have been observed exclusively in the core promoters of the developmentally regulated genes *aba*A, *brl*A and *trp*C from *A. nidulans*. These Inr-like elements are located 11 and 13 bp upstream of the tsp of respectively the *aba*A and *trp*C gene. In both genes the Inr-like element and tsp are sufficient for accurate transcription initiation and developmental regulation. Therefore, it was proposed that these fungal Inr-like sequences are functionally related to the mammalian Inr (Adams and Timberlake, 1990). An argument against the latter statement would be that, in contrast to the mammalian Inr, the fungal Inr-like element does not encompass the tsp.

Like mammalian and *S. cerevisiae* core promoters, the filamentous fungal core promoters tend to be small. In most cases only 50 bp upstream of the ATG are sufficient for transcription. The promoter of the *A. nidulans* 3'-phosphoglycerate kinase (*pgk*A) gene is a notable exception to this rule. In the *pgk*A promoter, sequences between 161 and 120 bp upstream of the tsp are indispensable for transcription. Additionally, data from experiments with different fusions of the *pgk*A gene to the *lac*Z reporter gene, suggest that transcription activating elements could even reside within the *pgk*A coding region (Streatfield *et al.*, 1992). Again, this is not a unique feature of filamentous fungal promoters, for similar observations have been made for the *pgk*A homologue in *S. cerevisiae* (Mellor *et al.*, 1987) and for several genes in *D. melanogaster* (Thummel, 1989).

The fact that not all promoters contain CT-boxes, TATA-, or Inr-like elements, suggests that, like in higher eukaryotes, there could be distinct classes of core promoters in filamentous fungi. To determine to what extent filamentous fungal core promoter elements resemble functionally their mammalian and *S. cerevisiae* homologues, detailed analysis is necessary.

Strategies for detecting upstream activating or repressing sequences in Aspergilli

The main objective in most promoter deletion studies has been the identification of gene specific *cis*-acting elements rather than elements governing basal level transcription in filamentous fungi. In most promoter studies the 5' proximal regions were either fused to the *E. coli lac*Z or *uid*A reporter gene. Subsequently, the effects of *in vitro* constructed mutations on promoter activity were monitored by respectively ß-galactosidase or ß-glucuronidase activity assays. In the case of *A. nidulans arg*B (Goc and Wegelenski, 1988) and *A. niger gla*A (Fowler *et al.*, 1990) the enzymatic activity of the gene product itself was used to monitor transcription.

A disadvantage of promoter deletion studies is, that the size of the deletions itself is generally an order of magnitude larger than the *cis*-acting elements they seek to identify. Consequently, such a promoter analysis can only provide an approximate location of *cis*-acting elements involved in repression

or stimulation of transcription. For instance, in the analysis of the Taka-amylase A (*amy*B) promoter from *A. oryzae* 100 bp deletions were used. This analysis allowed only an approximate location of the *cis*-acting element required for high level expression and starch induction of *amy*B (Tsuchiya *et al.*, 1992).

The "resolution" of a promoter analysis can be significantly enhanced in the case where the nucleotide sequence of the promoter of the same gene from a related species or of different genes, known to be similarly regulated, are available. In such a case, alignment of the 5' proximal regions can help to identify putative *cis*-acting elements on the basis of sequence homology. The functionality of such elements can then be assessed by site specific mutagenesis. The use of such a strategy, allowed the localisation of two *cis*-acting elements responsible for high level expression and maltose induction of the *A. niger* and *A. oryzae gla*A genes encoding glucoamylase (Fowler *et al.*, 1990: Hata *et al.*, 1992). In the case of the *A. nidulans gpd*A (Punt *et al.*, 1990) and *oli*C (Turner *et al.*, 1989) promoters, alignment with the sequences of their *A. niger* homologues permitted the deletions and site specific mutations to be planned in advance. With this strategy the *gpd*-box was identified as an important *cis*-acting element in the *A. nidulans gpd*A promoter.

In some cases, even comparison with more distantly related species like *S. cerevisiae* might give some useful information. For example in the *A. nidulans arg*B gene, data from a promoter deletion study, indicated the presence of regulatory elements 150 to 50 bp upstream of the tsp. This region contained three copies of target sequence of the *S. cerevisiae GCN4* gene product, involved in general control of amino acid biosynthesis (Goc and Weglenski, 1988). Unfortunately, no specific mutagenesis of these putative *GCN4* homologue binding sequences was performed in order to verify their functionality.

In genetically well characterised systems, like the *A. nidulans* nitrate utilisation genes, ethanol utilisation genes and the *amd*S gene, rapid progress is being made. This is possible, because extensive genetic and biochemical analysis have provided an outline of the regulatory circuits to which these genes are subject. Secondly, for all of these systems a wealth of mutants in both structural and regulatory genes is available.

Genetical and biochemical analysis already had established the involvement of the *alc*R gene product in the regulation of the ethanol utilisation genes *ald*A and *alc*A. Comparison of the *ald*A, *alc*A and *alc*R 5' proximal regions lead to the identification of putative binding sites for the *alc*R gene product (Felenbock, 1988). Later DNAseI protection assays confirmed that the identified sequences coincided with the ALCR footprint (Kulmburg *et al.*, 1992). The putative binding sites for *cre*A, mediating carbon catabolite repression in *A. nidulans* (Bailey and Arst, 1975), were initially identified by alignment of promoters known to be subject to catabolite repression (Fehlenbok *et al.*, 1989). Subsequent DNAseI protection assays demonstrated that these sequences bind the CREA protein *in vitro* (Kulmburg *et al.*, 1992). Proof that these CREA footprints represented functional CREA-binding sites *in vivo*, was obtained by the disruption of such a site in the *alc*R promoter. Disruption of this *alc*R CREA-binding site lead to *alc*R and *alc*A expression under repressing conditions (Kulmburg *et al.*, 1992). Also two operator mutations, resulting in carbon derepressed expression of the proline permease gene *prn*B, were shown to alter sequences encompassing the CREA footprint (Sophianopoulou *et al.*, 1993).

Mutations in the regulatory genes *amd*R, *amd*A, *fac*B, *cre*A and *are*A helped to define five regulatory circuits involved in *amd*S expression (Hynes *et al.*, 1989). Despite this multitude of complex regulatory circuits, most of their putative binding sites are confined to a region of 200 bp upstream of the tsp (Hynes *et al.*, 1989). The approximate locations of these *cis*-acting elements were determined through analysis of selected or *in vitro* constructed *amd*S promoter mutations. By introducing into *A. nidulans* multiple copies of oligonucleotides, that span the approximate location of the AMDR-binding site, the binding site was identified. In this *in vivo* titration experiment, oligonucleotides that contain the

AMDR-binding site sequester the AMDR protein and inhibit AMDR regulation of the resident *amd*S gene.

The regulatory role of the *nir*A and *are*A gene products in the expression of the nitrate utilisation genes of *A. nidulans* was also detected by classical genetic analysis (for a review see Cove, 1979). Like the quinic acid utilisation genes of *A. nidulans* and *N. crassa* (Hawkins *et al.*, 1989) exhibit their nitrate utilisation genes a similar organisation. Both *nir*A and *are*A have functionally homologous counterparts in *N. crassa*. The *nit-2* gene has been shown to substitute for most of the *are*A functions in *A. nidulans* (Davis and Hynes, 1987). Furthermore, the *nit-2* gene product protects specific sequences in the *A. nidulans nia*D promoter (Fu and Marzluf, 1990). Both the structural genes and the regulatory genes of the nitrate utilisation pathway have been cloned and characterised, opening the possibility for analysis at the molecular level. The deduced amino acid sequences of the NIRA (Burger *et al.*, 1991a/b) and AREA (Arst, 1989) encoding genes exhibited the characteristics of DNA-binding transcriptional activators. The analysis of the intergenic region of the divergently transcribed *A. nidulans nii*A and *nia*D genes revealed that NIRA binds to four in the *nii*A-*nia*D intergenic region, that fit the consensus CTC-CGHGG. Studies with reporter genes revealed, that the four NIRA-binding sites act bidirectionally, conferring nitrate induction to both genes (Punt *et al.*, 1995).

In recent publications the 5' proximal region of genes were scanned with mobility shift assays to detect putative promoter elements as an alternative to a promoter deletion study. In such a mobility shift analysis, a set of ^{32}P-labelled restriction fragments, spanning the 5' proximal region of the gene, are incubated both with and without crude extracts of nuclear proteins. Binding of proteins from the nuclear extract to some of these restriction fragments, can be detected by a reduction in the electrophoretic mobility of that fragment on a low ionic strength non-denaturing polyacrylamide gel (Baldwin and Sharp, 1987). The specificity of the binding between the restriction fragment and the protein can be verified by repeating the mobility shift in the presence of either a 10^5 molar excess of poly(dI.dC) or an excess of the unlabelled restriction fragment. If the protein-DNA interaction is specific, the presence of this excess of non-specific poly (dI.dC) DNA will not interfere with the mobility shift. By contrast, the excess of unlabelled restriction fragment will compete with its labelled counterpart and thus will suppress the mobility shift.

Initial attempts to study the promoter from the *Trichoderma reesei cbh*2 gene (encoding the cellulolytic enzyme cellobiohydrolase II) with a promoter-*E. coli uid*A fusion, failed because even under *cbh*2-inducing conditions ß-glucuronidase activity was just above background level. As an alternative, restriction fragments from the *cbh*2 promoter were used in mobility shift assays. In this way two putative *cis*-acting elements could be assigned to two restriction fragments. Because protein extracts from mycelia grown under *cbh*2 inducing and non-inducing conditions were used, one of the mobility shifts could be linked to induction (Stangl *et al.*, 1993).

The size of the fragments, used in mobility shift assays, limits the resolution of this method to a similar extent as the size of the deletions in a promoter deletion study. Therefore, like in a promoter deletion study, additional information is necessary for the characterisation of UASs and URSs. Another disadvantage of mobility shift scanning is that the assay is not a functional test for promoter activity.

The data of the analysis of the *N. crassa am* gene, encoding glutamate dehydrogenase, indicate that there might be a potentially more serious problem associated with the mobility shift scanning. For this analysis, restriction fragments, spanning the 5' proximal region of the *am* gene, were incubated with ammonium sulphate fractionated whole cell extracts. Band shifts were observed for the fragments that contained the three UASs, two of which had already been detected by genetical analysis. Nevertheless, from enzyme analysis it was known that *am* expression in *N. crassa* is subject to catabolite induction. Surprisingly, no promoter element was detected that conferred this catabolite induction to the *am* gene

(Chen and Kinsey, 1994). The authors do not offer an explanation why no such element was detected in the *am* promoter. It might be that either the catabolite induction is not regulated at transcription level or that the required *trans*-acting factor was not present in the ammonia sulphate fraction employed for the assay. If the latter explanation would be correct, mobility shift scans would not be a useful alternative to promoter deletion studies.

Scope of this study

The pathways for NADPH production in Aspergilli are still relatively unexplored, but studies in *A. nidulans* indicate that the two dehydrogenases of the pentose phosphate pathway play a pivotal role. In this study we attempt to elucidate the regulation of G6PD expression in relation with the NADPH/$NADP^+$ ratio. Although reports strongly suggests that control over G6PD expression in humans and rat is exerted also at other levels than transcription, this study addresses only transcriptional regulation. More specifically we try to identify the *cis*- and *trans*-acting elements in the promoters of the G6PD encoding genes from *A. niger* and *A. nidulans*. Our approach follows the strategy described above and hinges on the proposed close relation of *A. nidulans* and *A. niger*. This close relation would not only allow the identification of *cis*-acting elements by sequence comparison, but also extrapolation of the data on the regulation of the pentose phosphate pathway in *A. nidulans* to *A. niger*.

As a first step, described in chapter 2, we decided to purify the enzymes from *A. nidulans* and *A. niger* mycelium and to characterise them biochemically. Next, in chapters 3 and 4 the isolation and structural analysis of respectively the *A. niger* and *A. nidulans* G6PD encoding gene is described. In chapter 5 both the expression of the *A. niger* G6PD gene as the functional analysis of its promoter are described. Finally, in chapter 6 the regulation of G6PD encoding gene of *A. nidulans* by regulatory factors from the nitrate utilisation pathway is explored.

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Purification and characterization of glucose-6-phosphate dehydrogenase from *Aspergillus niger* and *Aspergillus nidulans*

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Glucose-6-phosphate dehydrogenase (G6PD; D-glucose 6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) has been purified from *Aspergillus nidulans* and *Aspergillus niger* by a combination of affinity and anion exchange chromatography. A 500–1000-fold purification was obtained and the final enzyme preparations were shown to be pure but not homogeneous. For both fungi the purified enzyme preparation gave two bands on native and denaturing gels. The catalytically active form is a multimer. The molecular mass of the monomers is 60 and 57 kDa for *A. nidulans* and 55 and 53 kDa for *A. niger*. Both enzymes exhibited strict specificity towards both substrates glucose 6-phosphate and $NADP^+$. The *A. nidulans* and *A. niger* G6PD enzymes catalyse the conversion of glucose 6-phosphate via a random order mechanism. Inhibition studies provided evidence for the physiological role of G6PD as producer of NADPH in both fungi.

Introduction

In fungi, the pentose phosphate and mannitol pathways are the central routes for the production of NADPH (Hult & Gatenbeck, 1978; McCullough *et al.*, 1977). The enzymes that generate NADPH are glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) of the pentose phosphate pathway and mannitol dehydrogenase of the mannitol pathway. G6PD catalyses the first reaction of the pentose phosphate pathway. The main physiological role of the pentose phosphate pathway is to provide NADPH, the reducing agent in a large number of biosynthetic and detoxification reactions (Levy, 1979). Under physiological conditions the oxidation of G6P by G6PD will be the committing step (Beutler & Kuhl, 1986).

G6PD is a ubiquitous enzyme, so widely distributed among prokaryotes and eukaryotes that it is considered a housekeeping enzyme (Levy, 1979). Detailed studies on this enzyme from filamentous fungi are scarce. Partial purification and characterization of the *A. niger* enzyme was reported by Jagannathan *et al.* (1956). Some characteristics of the enzymes from *Neurospora crassa* (Scott & Tatum, 1970; Scott & Mahony, 1976), *Penicillium duponti* and *P. notatum* (Malcolm & Shepherd, 1972), *Aspergillus parasiticus* (Niehaus & Dilts, 1984), *Agaricus bisporus* (Hammond, 1985) and *Phycomyces blakesleanus* (DeArriga *et al.*, 1986) have been published.

The activity of the pentose phosphate pathway in *A. nidulans* is influenced by growth conditions in order to maintain a proper NADPH:$NADP^+$ ratio (Carter & Bull, 1969; Bainbridge *et al.*, 1971; McCullough *et al.*, 1977; Singh *et al.*, 1988). Regulation of the NADPH:$NADP^+$ ratio is not only necessary to allow the use of certain carbon and nitrogen sources such as D-xylose and nitrate (Hankinson, 1974), but also affects the secondary metabolism. The NADPH:$NADP^+$ ratio has been shown to influence the type and relative amounts of polyols produced in both *A. niger* and *A. nidulans* (Dijkema *et al.*, 1985, 1986) as well as the biosynthesis of polyketides in the fungi *Alternaria alternata* (Hult & Gatenbeck, 1978) and *A. parasiticus* (Niehaus & Dilts, 1984).

To study the regulation of the NADPH/$NADP^+$ balance in aspergilli at the molecular level, we aimed to isolate and characterize both the enzymes and the genes governing decisive steps in the generation of NADPH. With the aid of the molecular genetic manipulation techniques developed for aspergilli (for a review see Goosen *et al.*, 1992) it will be possible to relate gene structure and regulation of gene expression to the physiology of the organism. Here we report the isolation

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Abbreviations: G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase.

and characterization of glucose-6-phosphate dehydrogenase from *A. niger* and *A. nidulans*.

Methods

Strains and growth conditions. A. nidulans WG 096 *yA2, pabaA1* and *A. niger* N402 *cspA1* (strain collection, Department of Genetics, Agricultural University, Wageningen) were used as a source for enzyme purification. Conidiospores were harvested in 0·05% Tween 80, 0·9% NaCl. After vigorous shaking the spore suspension was rinsed twice with 20 mM-potassium phosphate pH 2·0 for *A. niger* or pH 6·0 for *A. nidulans* and used to inoculate 600 ml of minimal medium (final concentration 2×10^7 spores ml^{-1}) (Pontecorvo *et al.*, 1953) with 2% (w/v) glucose. After shaking at 200 r.p.m. for 6 h at 37 °C for *A. nidulans* or 30 °C for *A. niger* the culture was added to 6·4 litres of the same medium in a 10 litre bottle. Cultures were heavily agitated by filter aeration for 25 h at 25 °C, a temperature favourable for the induction of glycolytic enzymes (Uitzetter, 1982). Mycelium was harvested by filtration through cheesecloth, washed with deionized water, blotted dry, frozen in liquid nitrogen and stored at −80 °C.

Preparation of the affinity matrices. The reactive dyes were coupled according to a modified procedure of Dean & Watson (1979). Sepharose CL-4B beads (Pharmacia) (200 g), extensively washed and suction filtered, were resuspended in 600 ml water and mixed with 4 g Cibacron Blue 3G-A (Ciba-Geigy). Following solubilization of the dye, the pH was raised to 10–11 by addition of 1 M-Na_2CO_3. The ionic strength was increased by addition of 120 ml 3 M-NaCl. Further treatment was as described by Uitzetter (1982). The reactive dye Procion Red P3 BN (ICI) was coupled to Sepharose CL-4B beads in a similar fashion.

Extraction and purification of the G6PD enzymes. All steps, except the FPLC runs, were carried out at 4 °C. Frozen mycelium (100 g) was powdered in liquid nitrogen, resuspended in 500–600 ml extraction buffer A (20 mM-potassium phosphate buffer pH 6·8, 1 mM-EDTA, 2 mM-$MgCl_2$ and 50 μM-PMSF) and stirred for 90 min. The cleared extract (20 min, 12000 *g*) was ammonium sulphate fractionated (15–65% saturation) and the precipitate was dissolved in 50 ml buffer A. After dialysis against buffer A the mixture was applied to a buffer A-equilibrated Cibacron Blue column (bed volume 100 ml), washed with 3 vols of buffer A and eluted with a linear gradient of 0–2 M-NaCl in buffer A. Active fractions, eluting at 0·8–1 M-NaCl, were pooled and dialysed overnight against buffer A. This dialysate was loaded on a buffer A-equilibrated FPLC-Mono Q HR 5/5 (Pharmacia) anion exchange column (bed volume 4 ml) at room temperature. The column was washed with buffer A and eluted with a non-linear gradient of 0–2 M-NaCl in buffer A; this gradient had a fairly flat slope from 0–1 M followed by a steep slope from 1–2 M. Active fractions eluting at 0·8–1·0 M-NaCl were pooled, dialysed against buffer A, reloaded on a buffer A-equilibrated FPLC-Mono Q HR 5/5 column and eluted with a non-linear 0–1·0 M-NaCl gradient (flat slope from 0–0·8 M-NaCl and a steep slope thereafter) in buffer A. Active fractions were again pooled, dialysed against buffer A and rechromatographed on a 5 ml Cibacron Blue column with a linear 0–2 M-NaCl gradient in buffer A. After pooling and dilution of the active fractions this last step was repeated. The G6PD-containing fractions were stored at 4 °C and for some purposes dialysed against buffer A and lyophilized in a Speedvac.

Enzyme assays. G6PD activity was measured at 30 °C in 50 mM-triethanolamine (pH 7·5), 5 mM-EDTA according to Löhr & Waller (1974). Under standard conditions the reaction mixture contained 790 μM-$NADP^+$ and 720 μM-G6P (Boehringer Mannheim). One enzyme unit is defined as the amount of enzyme required to reduce 1 μmol $NADP^+$ under the conditions specified. Effects of various compounds were examined by either incorporating them into the reaction mixture or preincubating the enzyme before adding substrates. Kinetic parameters were determined by least square regression; plotting of lines, replotting of intercepts and slopes were performed using Harvard Graphics.

Protein concentrations were determined by the Lowry method or using the Bio-Rad assay.

Polyacrylamide gel electrophoresis. Electrophoresis of native proteins was carried out in 10% (w/v) polyacrylamide gels at pH 8·9 as described by Davis (1964). Proteins were stained with Coomassie Brilliant Blue R-250. G6PD activity in native gels was detected as described by Shaw & Prasat (1970). SDS-PAGE was performed by the method of Laemmli (1970). Isoelectric focusing was performed in 5% polyacrylamide slab gels containing ampholytes spanning a 5–8 pH range, using the FBE 3000 apparatus (Pharmacia) according to a protocol supplied by the manufacturer. A standard mixture (Serva) of amyloglucosidase (pI 3·5), ferritin (4·4), bovine albumin (4·7), β-lactoglobulin (5·34), conalbumin (5·9), horse myoglobin (7·3), whale myoglobin (8·3), ribonuclease (9·45) and cytochrome *c* (10·65) was used to calibrate the gradient.

Production of antisera. Antibodies were raised in male New Zealand White rabbits by injecting purified *A. nidulans* or *A. niger* G6PD in Freund's complete adjuvant on day 1 and day 30. Antibody titres were checked 9 d after the second injection in an Ouchterlony agar diffusion test (Ouchterlony & Nilson, 1978). Two days later blood was collected, allowed to clot for 1–2 h at 25 °C, left overnight at 4 °C and spun to collect the antiserum. The antiserum was stored at −20 °C. Antibodies against a commercial preparation of *Saccharomyces cerevisiae* G6PD (Boehringer Mannheim) were kindly provided by H. C. M. Kester (Dept. of Genetics, Wageningen).

Western blotting and G6PD specific enzyme immunoassay. Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose (Schleicher and Schuell BA-85) by electroblotting in 25 mM-Tris, 192 mM-glycine (pH 8·3) and 20% (v/v) methanol for 3 h at 60 V (200 mA). Blots were blocked in 3% (w/v) gelatin in 20 mM-Tris/HCl (pH 7·5) and 0·5 M-NaCl. After extensive washing, the blots were incubated with G6PD antibody diluted in blocking buffer (1:250) at room temperature for at least 2 h with gentle agitation. Again the blots were washed and incubated with diluted (1:3000) goat anti-rabbit gamma-globin–alkaline phosphatase conjugate and finally stained with the Immune Blot assay kit according to the manufacturer's instructions (Bio-Rad). For the G6PD specific enzyme immunoassay, the G6PD antibody-incubated blots were washed in buffer A and subsequently incubated for at least 4 h at room temperature with 2–3 U of either *A. nidulans* or *S. cerevisiae* G6PD enzyme in buffer A. After removal of unbound G6PD the blots were stained for G6PD activity according to Shaw & Prasat (1970).

Determination of molecular mass by gel filtration. Gel filtration was carried out at 4 °C either on a 83 × 2 cm Sephacryl S-300 or a Sephacryl S-200 (Pharmacia) column equilibrated with buffer A. The column was calibrated using ferritin (450 kDa), catalase (240), aldolase (160) and bovine serum albumin (68).

Results

Enzyme purification

G6PD from *A. niger* and *A. nidulans* were purified by exactly the same procedure. Starting with 100 g of frozen mycelium, recoveries of 15–30% were usually obtained with an overall 500–1000-fold purification. Represen-

Table 1. *Purification of A. niger and A. nidulans glucose-6-phosphate dehydrogenases*

Purification step	Volume (ml)	Total activity* (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (-fold)
(*a*) *A. niger*						
Homogenate	500	458	803	0·57	100	1
$(NH_4)_2SO_4$ precipitation	65	803	450	1·8	175	3·2
Cibacron Blue 1	43	586	22·5	26	127	46
Mono Q 1	5	381	3·2	117	82	206
Mono Q 2	3	278	1·05	265	61	467
Cibacron Blue 2	4	195	0·35	556	42	976
(*b*) *A. nidulans*						
Homogenate	750	1507	1800	0·84	100	1
$(NH_4)_2SO_4$ precipitation	65	1541	906	1·7	102	2·0
Cibacron Blue 1	40	1028	46·4	22·2	68	26
Mono Q 1	3	767	3·0	256	51	304
Mono Q 2	3	743	1·5	495	49	589
Cibacron Blue 2	8·25	265	0·41	646	18	769

* 1 unit of G6PD activity is the amount of enzyme required to produce 1 μmol NADPH min^{-1} (mg protein)$^{-1}$.

tative examples of the purification of the *A. niger* and *A. nidulans* enzymes are presented in Table 1.

In the first step (ammonium sulphate fractionation) a recovery of more than 100% was consistently observed, presumably due to the removal of interfering material from the homogenate, resulting in some activation of the enzyme. In the affinity chromatography step some irreversible binding of G6PD to the Cibacron Blue matrix was observed. After the resin had been used once no more activity was lost. This step allowed the removal of large amounts of protein with excellent recovery of activity. Elution of the Cibacron Blue column with 100 μM-$NADP^+$ did not result in the release of bound enzyme. However pre-incubation of a purified enzyme preparation with $NADP^+$ before application resulted in reduced affinity for this matrix. In this respect, results are in accordance with those for yeast (Reuter *et al.*, 1986), but contrast with those for sea urchin (Matsuoka, 1988). Procion Red P3BN (Reuter *et al.*, 1986) was tested as an alternative matrix for enzyme purification; the affinity was much lower than for Cibacron Blue and consequently recoveries were poor. Also no elution of enzyme activity from this resin was observed with 100 μM-$NADP^+$.

Purity and homogeneity of the enzyme preparations

As judged from native acrylamide gels, both *A. niger* and *A. nidulans* G6PD enzyme preparations showed two bands when stained for protein. The same bands were also observed when the gels were stained for G6PD activity (data not shown). For *A. nidulans* one band was

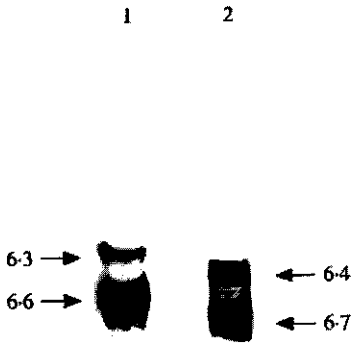

Fig. 1. Isoelectric focusing of purified glucose-6-phosphate dehydrogenase from *A. nidulans* and *A. niger*. IEF was performed in 5% (w/v) polyacrylamide gel slabs containing ampholytes for the pH range 5–8. Protein bands were visualized by staining for G6PD activity. pI values for the different enzyme forms as derived from a calibration standard are indicated. Lane 1, 0·8 U *A. nidulans* enzyme; lane 2, 0·3 U *A. niger* enzyme.

usually more prominent. Moreover the relative intensity of the two bands varied from one preparation to another. For *A. niger* these differences were less pronounced. Both purified G6PD preparations also separated into double bands with different intensities on 10% SDS-polyacrylamide gels.

In IEF gels (Fig. 1) double bands with similar intensities were observed. The deduced isoelectric points

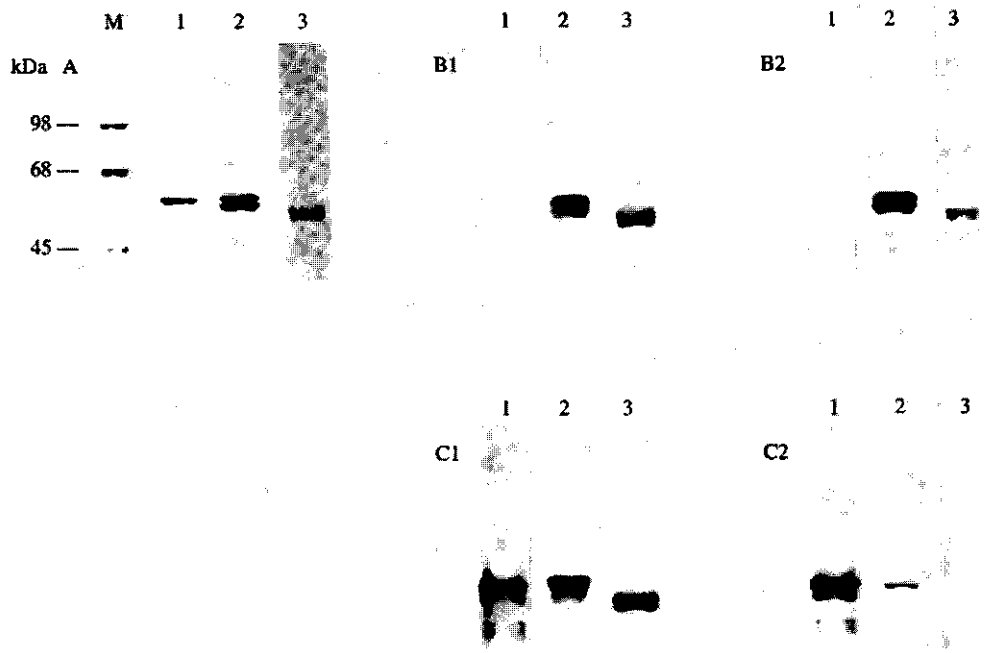

Fig. 2. Immunological detection of glucose-6-phosphate dehydrogenase protein bands from *A. nidulans* and *A. niger*. Denatured proteins were separated on SDS-polyacrylamide gels and either stained for protein (panel A) or used for Western blotting and immunochemical detection (panels B and C) as described in Methods. Panel A, Coomassie Brilliant Blue staining; panel B1, Western blot incubated with a polyclonal antiserum directed against *A. nidulans* G6PD and detection with alkaline phosphatase-conjugated second antibody; Panel B2, 'sandwich' blot incubated with a polyclonal antiserum raised against *A. nidulans* G6PD and detection by activity staining with *A. nidulans* G6PD; panel C1, as B1 but incubated with a polyclonal antiserum raised against yeast G6PD; panel C2, as B2 but incubated with a polyclonal yeast G6PD antiserum and detection by activity staining with yeast G6PD. Lanes 1, yeast G6PD; lanes 2, *A. nidulans* G6PD; lanes 3, *A. niger* G6PD; lane M, molecular mass markers: phosphorylase b (98 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa). The amounts of protein applied were, respectively, 2–4 μg in panel A, 1–2 μg in panels B1 and C1 and 0·2–0·4 μg in panels B2 and C2.

were 6·3 and 6·6 for *A. nidulans* and 6·4 and 6·7 for *A. niger*.

On Western blotting from 10% SDS-polyacrylamide gels the antiserum, raised against the *A. nidulans* enzyme preparation, reacted not only with the two *A. nidulans* bands but also with those of *A. niger* (Fig. 2, B1). Only a weak cross-reaction with the yeast G6PD was observed (Fig. 2, B1). Similarly, the *Aspergillus* doublets reacted with antibodies raised against the yeast G6PD, indicating that both bands are related to the yeast G6PD. The reaction of this yeast antiserum with the yeast G6PD enzyme preparation was much stronger and showed multiple degradation products (Fig. 2, C1) besides a major band.

G6PD specific detection of multiple monomers

To demonstrate that both bands visible in Coomassie-stained SDS-polyacrylamide gels and Western blots were implicated in G6PD activity, 'sandwich' blotting was devised. Western blots were incubated with an excess of *A. nidulans* antiserum and, after removal of unbound antibody, they were incubated with the homologous purified G6PD enzyme preparation. Antibody-bound G6PD was subsequently detected by activity staining.

As shown in Fig. 2, B2, both bands in the *A. nidulans* and *A. niger* enzyme preparations could be detected with the *A. nidulans* antiserum and enzyme. The yeast enzyme could not be detected with the *A. nidulans* antiserum/enzyme combination. The doublets in the *A. nidulans* preparation gave a stronger signal than the two bands in the *A. niger* preparation. Conversely with the *A. niger* G6PD antiserum and enzyme, the two bands in the *A. niger* preparation stained more strongly than the *A. nidulans* doublets (not shown). In a similar experiment with the yeast antiserum and enzyme preparation, the yeast bands stained heavily. Additionally, only one weak band was observed in the *A. nidulans* preparation and no signal was found for *A. niger* (Fig. 2, C2).

The specificity and sensitivity of the G6PD 'sandwich' Western blot were so high, that for *A. niger* as well as for *A. nidulans* the two monomeric forms could be detected in 15–30 μg protein of a crude extract. Non-specific bands, sometimes observed in normal Western blots,

were completely absent in the 'sandwich' blots. These results strongly indicate, that our final G6PD preparations were pure, although not homogeneous.

Molecular masses

From SDS-polyacrylamide gels (Fig. 2A), the molecular masses of the monomers involved in G6PD activity were estimated to be 55 and 53 kDa for *A. niger* and 60 and 57 kDa for *A. nidulans*. The molecular mass of the *S. cerevisiae* G6PD monomer was estimated to be 59 kDa.

The elution profiles of Sephacryl S300 as well as S200 gel filtration columns indicated a molecular mass for the native enzyme of 160 kDa for *A. niger* and 180 kDa for *A. nidulans*. G6PD activity could not be detected in other column fractions, which suggests the absence of active monomers and dimers.

Kinetics and substrate specificity

For both *Aspergillus* enzymes a broad pH vs. initial velocity curve was obtained, with an optimum ranging from pH 7·0 to 9·0 (not shown). Under standard assay conditions the relationship between the amount of enzyme added and the initial reaction velocity was linear. Within this linear range the reaction parameters were analysed.

The *A. nidulans* and *A. niger* enzymes exhibited absolute specificity towards the substrates G6P and $NADP^+$. No activity was observed with NAD^+ (up to 1 mM), glucose, glucose 1-phosphate, 2-deoxyglucose 6-phosphate, fructose, fructose 6-phosphate, fructose 1,6-diphosphate, mannose or galactose 6-phosphate (up to 600 μM).

With the two substrates normal hyperbolic saturation curves were obtained and the double reciprocal plots were linear. The $1/V$ vs $1/[G6P]$ plots intersected at a common point above the abscissa. The kinetic parameters of *A. nidulans* and *A. niger* were deduced from Eadie–Hofstee plots of the data of the initial velocity experiments. Fig. 3 shows an example for the *A. nidulans* enzyme. The kinetic parameters are summarized in Table 2.

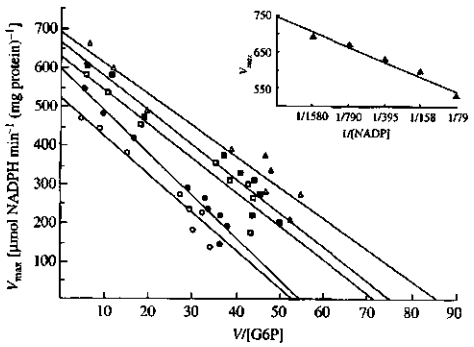

Fig. 3. Kinetics of *A. nidulans* glucose-6-phosphate dehydrogenase. Eadie–Hofstee plot: initial velocity vs the ratio of initial velocity and glucose-6-phosphate concentrations, determined at several fixed $NADP^+$ concentrations. From the slopes of these plots the K_m for G6P was calculated and from replots of the intercepts with the ordinate vs $1/[NADP^+]$ the maximal velocity was deduced (see insert). Assays were done at 30 °C with 0·05 U *A. nidulans* G6PD. Velocities are expressed as μmol NADPH min^{-1} (mg protein)$^{-1}$. ○, 79 μM-$NADP^+$; ●, 158 μM-$NADP^+$; □, 395 μM-$NADP^+$; ■, 790 μM-$NADP^+$ and △, 1580 μM-$NADP^+$.

The effect of NADPH on the initial reaction velocity was studied in more detail at several fixed concentrations of one substrate and a variable concentration of the other. For both enzymes an almost ideal competitive inhibition was observed with respect to $NADP^+$. For G6P this was less clear, although competition was apparent. The K_i(NADPH) value for the *A. nidulans* enzyme preparation was deduced and shown to be in the same order as the K_m for $NADP^+$. Because of the instability of the second reaction product, 6-phosphoglucono-δ-lactone, it was not tested. Under normal physiological conditions this compound might be expected to be removed immediately (Beutler & Kuhl, 1986). The effect of specific compounds, known to influence G6PD from other sources, e.g. AMP, ADP, ATP, palmitoyl-CoA, pyridoxal-5-phosphate and acetylsalicylic acid on the initial reaction velocity was investigated under standard conditions and in some

Table 2. *Kinetic parameters for A. nidulans and A. niger G6PD enzymes*

	K_m(G6P) (μM)	K_m(NADP) (μM)	K_i(NADPH) (μM)	V_{max} [μmol NADPH min^{-1} (mg protein)$^{-1}$]
A. niger	153±10	26±8	ND	790
A. nidulans	92±10	30±8	20±5	745

ND, Not determined.

cases at a range of substrate concentrations. No significant effects were observed. High ATP concentrations (> 2–4 mM) inhibited the enzyme slightly, but inhibition was not related to the NADPH:$NADP^+$ ratio as in *Candida maltosa* H (Röber *et al.*, 1984).

Discussion

In this paper we report on a fast and efficient purification of glucose-6-phosphate dehydrogenase from two *Aspergillus* species. This procedure can be completed in 2 d and results in pure preparations with high specific activities. This is mainly due to the use of the affinity dye Cibacron Blue which, because of its high and specific binding capacity for G6PD, is effective in removing large amounts of contaminating proteins.

Final preparations showed double bands in both native and isoelectric focusing gels, each of which represented an enzymic form of G6PD as judged by activity staining in gels. The presence of multiple G6PD enzymes is not unprecedented since multiple bands have been observed in bacteria, plants, animals and insects. In general it used to be assumed that different multimers of one basic subunit were responsible for this phenomenon (Levy, 1979). The observation of double bands in the SDS-polyacrylamide gels suggests that there could be two separate monomeric forms of G6PD in aspergilli. Although not excluding the possibility of contamination, detection of two bands in both preparations on Western blots, irrespective of the antiserum used, supports this suggestion.

The cross reaction of the *A. nidulans* antiserum is much stronger with the *A. niger* G6PD than with the *S. cerevisiae* G6PD. This strongly suggests that the two *Aspergillus* G6PDs are more closely related to each other than to *S. cerevisiae* G6PD. Definite proof for the G6PD specificity of the monomers was obtained using the 'sandwich' blot. In this procedure the divalent nature of immunoglobins is exploited. After binding of the antibody to the denatured protein on the Western blot, some of the antigen binding sites will still be available for the binding of native G6PD, provided that the protein on the blot is at least related to G6PD. Using a G6PD activity stain only G6PD specific antibodies are detected. Application of this technique is by no means limited to G6PD but can be used to detect any enzyme immunologically as long as a specific activity stain is available.

Combining these results with the data from non-denaturing and isoelectric focusing gels, we speculate that in aspergilli two G6PDs with multimeric structures consisting of two different monomers are present. Our gel filtration data are in agreement with the molecular mass of 190 kDa, previously obtained for partially purified *A. nidulans* G6PD (Malathi & Shanmugasundaram, 1987) and suggest a trimeric structure for the active forms from both fungi. However dimers and tetramers tend to be the rule for active G6PDs (Bonsignore & De Flora, 1972; Levy, 1979). Retardation by interaction with Sephadex G-200, resulting in an underestimation of the molecular mass, has been reported for G6PD of *A. parasiticus* and *S. cerevisiae* by Niehaus & Dilts (1984). To resolve the question whether the native G6PD is indeed a tetramer, SDS-PAGE of chemically cross-linked subunits might be an alternative.

Because of the small differences in molecular mass and the limited resolution of gel filtration, we cannot tell whether the G6PDs are homomeric or heteromeric structures. Assuming a tetrameric structure for both G6PDs, the observation of only two and not five bands on non-denaturing gels points towards a highly specific aggregation. The variability of relative intensities of the bands observed between different isolates can be used as an argument for proteolytic degradation, post-translational modification or even different subunits derived from two genes.

Aspergilli are known to produce substantial amounts of various proteases (van den Hondel *et al.*, 1992) and so heterogeneity caused by limited proteolysis cannot be excluded, even though PMSF was included in all buffers at all stages. Since PMSF and EDTA inhibit only specific classes of proteases, the chance of proteolytic degradation during isolation could be reduced by employing cocktails of protease inhibitors. In this respect it is interesting to note that the multiple subunit composition reported for the *arom* multi-enzyme complex from *N. crassa* was shown to be the result of proteolytic degradation during isolation. If protease activity is stringently controlled during isolation the *arom* multi-enzyme complex can be shown to be composed of two, probably identical, subunits (Lumsden & Coggins, 1977).

Post translational modification of G6PD has been reported for several cell types in humans in relation to differentiation, aging and disease (Levy, 1979). The isolation of a 'G6PD modifying factor' from human leukaemic granulocytes has been reported (Kahn *et al.*, 1976). The exact nature of these modifications however has not been elucidated, but they did not alter the molecular mass of the enzyme.

In *Acetobacter hansenii* two different G6PD enzymes are encoded by separate genes. These enzymes however, have a distinct cofactor specificity: one is NAD^+ specific and the other is $NADP^+$ specific (Levy & Cook, 1991). This cannot be related directly to the double bands observed in both *Aspergillus* enzyme preparations, since they were shown to exhibit absolute specificity towards $NADP^+$.

Interestingly the $NADP^+$-specific G6PD from the fungus *N. crassa* also shows multiple bands on native

and isoelectric focusing gels at all stages of purification. Although only one band with a molecular mass of 57 kDa is observed on SDS-polyacrylamide gels, mutations in four unlinked genes lead to G6PD enzymes with altered electrophoretic and catalytic properties (Scott & Tatum, 1970; Scott & Mahony, 1976; Nishikawa & Kuwana, 1985). Though definite proof has never been obtained, these four genes are thought to represent structural genes for G6PD (Scott & Mahony, 1976).

In *Aspergillus oryzae* two G6PD species are present in glucose-grown mycelium and a third one is specifically induced during growth on ribose. These G6PD enzymes have distinct catalytic properties and molecular masses of 92, 117·5 and 141 kDa (Muiño Blanco *et al.*, 1983; Cébrian-Pérez *et al.*, 1989). Since no SDS-polyacrylamide gel data were presented by the authors it is difficult to assess whether the observed enzyme forms are due to different aggregation states or to different types of subunits.

The Michaelis constants for *A. nidulans* G6PD differed from the ones determined [K_m(G6PD) 20 μM and K_m(NADP$^+$) 77 μM] by Malathi & Shanmugasundaram (1987) for a partially pure preparation. The value for the K_m(G6PD) (170 μM) for a partially purified *A. niger* G6PD determined by Jagannathan *et al.* (1956) agrees with our data. The K_m and V_{max} for both *Aspergillus* G6PDs were in the same range as those reported for *P. duponti*, *P. notatum*, *A. bisporus*, *S. cerevisiae*, *Candida maltosa* H and both G6PDs from *A. oryzae* present in mycelium grown on glucose but differed markedly from those reported by Niehaus & Dilts (1984) for the G6PD isolated from *A. parasiticus*.

The kinetic data for both enzyme preparations are similar to those obtained with enzyme preparations from other sources (Bonsignore & De Flora, 1972; Levy, 1979), e.g. absolute specificity for both substrate (G6P) and cofactor (NADP$^+$), high affinity for both substrates and strong competitive inhibition by NADPH. The double reciprocal plots of the initial velocity data have an intersecting pattern of lines, which rules out the possibility of a ping-pong mechanism. Since NADPH is a competitive inhibitor with respect to both NADP$^+$ and G6P, a rapid equilibrium random mechanism fits our data best. A similar mechanism has been proposed for G6PD from *P. duponti*, *P. notatum* (Malcolm & Shepherd, 1972), and *Saccharomyces carlsbergensis* (Holten *et al.*, 1976). For G6PD from human, pig and rat liver an ordered mechanism has been deduced from inhibition studies (Levy, 1979).

The high ATP concentration needed for inhibition and the absence of any correlation with the NADPH:NADP$^+$ ratio make it questionable whether this inhibition has any physiological significance. Such a correlation was clearly demonstrated for *C. maltosa* H, where the pentose phosphate pathway serves as an alternative for glycolysis to generate glyceraldehyde 3-phosphate (Röber *et al.*, 1984). This supports the view that in aspergilli the pentose phosphate pathway is mainly involved in providing NADPH for anabolic purposes. In general it can be stated that in both *Aspergillus* species the G6PD activity is regulated by the NADPH:NADP$^+$ ratio.

Since reactive lysine residues, which can be modified by pyridoxal-5-phosphate (Vincenzini *et al.*, 1986; Camardella *et al.*, 1988) and acetylsalicylic acid (Han *et al.*, 1980; Jeffery *et al.*, 1985) occupy similar positions in G6PD enzymes of widely different origin, the absence of any effect of these compounds on the *Aspergillus* enzymes is surprising. The result appears particularly peculiar since sequence analysis of the G6PD encoding genes recently isolated from *A. niger* and *A. nidulans* showed that such a lysine is present in both enzymes in a highly conserved sequence context (P. J. H. van den Broek and others, unpublished results).

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Chapter 3 Isolation and characterisation of the glucose-6-phosphate dehydrogenase encoding gene (*gsd*A) from *Aspergillus niger*.

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Summary

Genomic and cDNA clones encoding glucose-6-phosphate dehydrogenase (G6PD) were isolated from the fungus *Aspergillus niger*. Sequence analysis of the glucose-6-phosphate dehydrogenase gene (*gsd*A) revealed an open reading frame of 1530 bp, encoding a protein of 58.951 kD. The *gsd*A gene is interrupted by nine introns; one exceptionally large intron (348 bp) and eight introns of normal fungal dimensions (50-100 bp). The region upstream of the ATG contains several C+T rich stretches, direct and inverted repeats but no clear TATA or CCAAT boxes can be found. *A. niger* strains overproducing G6PD were constructed by cotransformation of *gsd*A subclones. Overexpression of G6PD in cotransformants was shown to be deleterious for the fungus, especially when they were grown on media containing ammonia. Attempts to construct a *gsd*A null mutant by gene disruption were unsuccessful.

Introduction

Filamentous fungi like *Aspergillus niger*, *Aspergillus nidulans* and *Neurospora crassa* are excellent model systems to study the regulation of metabolic pathways, because of the well developed genetics and the availability of large numbers of characterised mutants. Extension of genetic and biochemical analysis with the tools of recombinant DNA technology allows dissection of metabolic pathways and analysis of their regulation at the molecular level. This approach has been successfully used to study the regulation of the *A. nidulans amd*S gene (Hynes *et al.*, 1989), the nitrate assimilation genes in *A. nidulans* (Unkles, 1989) and *N. crassa* (Fu and Marzluf, 1988), the quinic acid utilisation genes in *A. nidulans* (Hawkins *et al.*, 1989) and *N. crassa* (Giles *et al.*, 1985) and the ethanol utilisation regulon in *A. nidulans* (Felenbok *et al.*, 1989).

Our aim is to use such an integrated approach to study the regulation of glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) expression in *A. niger* and *A. nidulans*.

G6PD catalyses the first and probably committing step of the pentose phosphate pathway (Beutler and Kuhl, 1986). The oxidative branch of this pathway is considered to be the major source of cellular

NADPH, the reductive agent in many biosynthetic and detoxification reactions (Levy, 1979). The non-oxidative branch generates pentoses for the biosynthesis of nucleic acids and aromatic amino acids (Peleato *et al.*, 1991).

The wide array of carbon and nitrogen sources *A. niger* is able to metabolise, makes the regulation of its G6PD expression particularly interesting. The demand for NADPH varies, depending on the carbon and nitrogen source used: D-xylose, L-arabinose and nitrate need to be reduced with NADPH in order to be metabolised (Unkles *et al.*, 1989; Witteveen *et al.*, 1990). During growth on D-xylose, L-arabinose and nitrate, *A. niger* has to tune its G6PD expression to meet the increased NADPH demand. An increase in G6PD activity in response to growth on D-xylose or L-arabinose was demonstrated in *A. niger* by Witteveen *et al.* (1990).

As a first step in our attempt to study the regulation of glucose-6-phosphate dehydrogenase expression, we isolated and characterised the enzyme from mycelium of *A. niger* and *A. nidulans* (Wennekes *et al.*, 1993). Both pure enzyme preparations were shown to consist of two bands on native and denaturing polyacrylamide gels. The molecular weight of the *A. niger* monomers, estimated from SDS-polyacrylamide gels, is 55 and 53 kD. The *A. niger* monomers cross react strongly with *A. nidulans* G6PD antiserum. Evidence that both monomers in *A. niger* and *A. nidulans* are involved in G6PD activity was obtained by G6PD specific sandwich immunoblotting (Wennekes *et al.*, 1993). The active form of the G6PD enzyme is a multimer, most probably a tetramer. The two bands in native polyacrylamide gels suggest that the two monomeric forms aggregate to two different multimers.

These two monomers could be the result of proteolytic degradation or post-translational modification of one basic subunit. Alternatively, the monomers could be derived from two different G6PD encoding genes. In humans (Persico *et al.*, 1986: Chen *et al.*, 1991), rat (Ho *et al.*, 1988), *D. melanogaster* (Hori *et al.*, 1985: Fouts *et al.*, 1988) and *Saccharomyces cerevisiae* (Nogae and Johnstone, 1990: Thomas *et al.*, 1991) G6PD has been shown to be encoded by a single gene. To resolve this issue and to have the advantage of the use of DNA manipulation techniques, we decided to clone and characterise the G6PD encoding gene from *A. niger*.

Materials and methods

All chemicals used were analytical grade whenever commercially available. Radiochemicals were purchased from Amersham.

Plasmids, strains, growth conditions and transformation

The plasmids pGW635 (Goosen *et al.*, 1985), pGW101 (Wernars *et al.*, 1985), pAB2-5 (Kos *et al.*, 1985), pAN7-1 (Punt *et al.*, 1987), pRS4 (Smit *et al.*, 1992) pEMBL-18/19 (Dente *et al.*, 1983) and pTZ-18u/19u (USB) were used. λ clone D_mG6PD14 (Hori *et al.*, 1985) containing the *D. melanogaster* G6PD gene was kindly provided by dr. S.H. Hori. The 3.9 kb *Eco*RI fragment of λD_mG6PD14 was subcloned in pUC19 (Yanisch-Perron *et al.*, 1985) to give pGW175. The 1.0 kb *Eco*RI-*Pst*I fragment of PGW175 was used to screen the *A. niger* genomic library.
For transformation and propagation of plasmids *E. coli* DH5α (λ$^-$, F$^-$, *end*A1, *hsd*R17 (r_k^- m_k^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, φ80Δ*lac*ZM15) was used (Sambrook *et al.*, 1989). *E. coli* LE 392 (F$^-$, λ$^-$,

*hsd*R514 (r_k^- m_k^+), *sup*E44, *sup*F58, *lac*Y1, *gal*K2, *gal*T22, *met*B1, *trp*R55) was used as host for recombinant λ phages (Murray *et al.*, 1977; Sambrook *et al.*, 1989).
The *A. niger* N400 wild type strain and its derivative N402 *csp*A1 (Bos *et al.*, 1987) were respectively used for the construction of a cDNA library and a genomic library. N593 *csp*A1, *pyr*A6 (Goosen *et al.*, 1985) and N755 *csp*A1, *pyr*A6, *nic*A1 (Bos *et al.*, 1988) were used for transformation experiments. *A. niger* was grown on minimal medium (MM), complete medium (CM) and supplemented minimal medium (SM) as described by Pontecorvo *et al.* (1953).

For transformation *A. niger* N593 was grown overnight at 30°C in MM, supplemented with 0.2% casamino acids, 0.5% yeast extract and 10 mM uridine. For *A. niger* N755 also 1μg/ml nicotinamide was added. The mycelium was harvested by sterile filtration, rinsed once with water and twice with 20 mM MES (pH 5.8), 1.33M sorbitol and 50 mM $CaCl_2$ ($S_{1.3}$MC). Approximately 0.5 g mycelium was resuspended in 20 ml of a filter sterilised solution of 5 mg/ml Novozyme 234 (Novo Industries, Denmark) in $S_{1.3}$MC and incubated at 30°C for 2 to 4 hours with gentle agitation. The protoplasts were freed from adhering mycelium by pipetting up and down. The protoplasts were purified by filtration over a loose glasswool plug in a funnel and collected by centrifugation for 10 min at 3000 rpm. The protoplasts were washed twice with 25 ml of 10 mM Tris (pH 7.5), 1.33 M sorbitol and 50 mM $CaCl_2$ ($S_{1.3}$TC), counted and resuspended to give a final concentration of 10^8 protoplasts/ml. 2.10^7 protoplasts were mixed with a maximal volume of 20 μl of transforming DNA and 50 μl 25% polyethylene glycol 6000 (BDH) in 10 mM Tris (pH 7.5), 50 mM $CaCl_2$. After a 20 min incubation on ice another 2 ml of 25% polyethylene glycol 6000, 10 mM Tris (pH 7.5), 50 mM $CaCl_2$ were added. After 5 min incubation at room temperature the transformation mixture was diluted with 4 ml of $S_{1.3}$TC. Aliquots of 1 ml were mixed with 4 ml topagar, prewarmed to 45°C, and immediately plated onto petridishes containing 20 ml of solid medium. Both the topagar and the solid medium in the plates consisted of MM with 2% agar, stabilised with 1 M sucrose. For the selection of a *gsd*A null mutant 200 μg/ml hygromycin B, 10 mM uridine, 0.2 mg/ml methionine and 0.8 mg/ml 5-fluoroorotic acid (FOA) were added to CM.

Isolation and manipulation of nucleic acids

General manipulations of nucleic acids were performed according to Sambrook *et al.* (1989). Dideoxy-sequencing (Sanger *et al.*, 1977) was performed using T7 and Taq DNA polymerase sequencing kits (Promega and USB) on ds-templates according to the protocols from the suppliers. Gel purified restriction fragments were labelled by the random primed labelling method (Feinberg and Vogelstein, 1983) with a kit from Boehringer Mannheim according to the suppliers' protocol or as described by Sambrook *et al.* (1989).

For the isolation of chromosomal DNA, mycelium was frozen in liquid nitrogen and ground in a micro dismembrator II (Braun, FRG). The powder was transferred to a 12 ml polypropylene tube containing a prewarmed mixture (55°C) of 1.5 ml water saturated phenol, 1 ml of a freshly prepared solution of 20 mg/ml triisopropyl-naphthalene sulphonic acid (Kodak Chemical Co.), 1 ml of 120 mg/ml 4-amino-salicylic acid and 0.5 ml 0.5 M Tris-HCl (pH 8.5), 0.5 M NaCl, 0.1 M EGTA. The tubes were vigorously mixed at 55°C every 30 seconds for 5 minutes. One ml chloroform-isoamylalcohol (24:1 v/v) was added and the tubes were again vigorously mixed for two minutes. Subsequently the tubes were spun in a tabletop centrifuge for 10 min at 3500 rpm. The aqueous phase was extracted once with phenol-chloroform-isomylalcohol (25:24:1 v/v) and twice with chloroform-isoamylalcohol. The DNA was precipitated by adding two volumes of ethanol.

For Southern analysis Qiagen tube 20 were used for further purification as prescribed by the manufacturer (Diagen). λ DNA was purified with Qiagen tip 20 according to the manufacturer's protocol.
For the construction of the cDNA library total RNA was extracted from powdered mycelium as described by Cathala (1983). RNA for primer extension and S1 assays was isolated as described by van Kan (1991).

Primer extension and S1 mapping

3 pmol of an oligonucleotide 5'-CCTCAGTGCGTGCTATTGTGCTGGCC-3' were end labelled with 15μCi of ^{32}P-γ-ATP (3000 Ci/mM) and 10 U T4 polynucleotide kinase (BRL) in 10 μl 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT and 0.1 mM spermidine. After 1 hour of incubation at 37°C, the kinase was heat-inactivated at 100°C for 10 min and 0.75 pmol labelled primer was added to a 20 μl reaction mixture containing 20 μg of total Aspergillus RNA, 20 mM Tris-HCl (pH 8.8), 2 mM of all dNTP's, 15 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 0.05% Tween20, 0.05% Nonidet NP40 and 4 U TET-z DNA polymerase (Amersham). The reaction mixture was covered with silicone oil and subjected to an initial 12 min denaturation at 95°C, 10 min annealing at 50°C, 10 min elongation at 60°C, followed by 30 cycles of 2 min denaturation at 95°C, 10 min annealing at 50°C. and 10 min extension at 60°C in a thermal cycler (Hybaid). A sample of the reaction mixture was run on a standard sequencing gel, next to a sequence reaction primed with the same oligonucleotide.

For S1-mapping 2 μg pGW259 (fig.1) was denatured in 40 μl 0.2 M NaOH, 2 mM EDTA for 5 min, neutralised with 4μl 2 M NH_4Ac (pH 4.6) and precipitated with 2.5 volumes of absolute ethanol. The probe was prepared by annealing 4 pmol of the aforementioned oligonucleotide to the alkali denatured plasmid for 10 min at 37°C and extension was performed in 90 mM Hepes (pH 6.6), 10 mM $MgCl_2$, 2 mM DTT, 50 μM dGTP, dTTP, dCTP and 50 μCi ^{32}P-α-dATP (3000 Ci/mM) for 30 min at room temperature with 6 U Klenow DNA polymerase (BRL). The reaction was chased by addition of 1mM dATP for 30 min at room temperature. Subsequently the Klenow polymerase was heat-inactivated and the probe was digested with 20 U *Hind*III for 30 min at 37°C. The probe was purified on a standard sequencing gel and eluted according to Sambrook *et al.* (1989). The probe and 50 μg total RNA were dissolved in 30 μl 40 mM Pipes (pH 7.0), 1 mM EDTA, 0.1% SDS and denatured for 5 min at 85°C and hybridisation was performed at 60°C for 16 hours. Subsequently 300 μl 0.05 M NaAc (pH 4.5), 0.28 M NaCl, 4.5 mM $ZnSO_4$ and 800 U/ml S1 nuclease was added to the hybridisation mixture and incubated for 1 hour at 37°C. The reaction was stopped by addition of 80 μl of 4M NH_4Ac, 50 mM EDTA (pH 8.0) and 50 μg/ml yeast RNA (Boehringer Mannheim) and the S1 resistant nucleic acids were precipitated with 2 volumes of ethanol. The pellet was washed twice with 70% ethanol, dried and dissolved in 4 μl sterile water. After addition of 4 μl 95% formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanole the samples were denatured for 5 min at 100°C. After cooling on ice the samples were loaded onto a standard sequencing gel next to a sequencing reaction, primed with the same oligonucleotide as was used to synthesise the probe.

Libraries and screening

The *A. niger* N402 genomic library (Goosen *et al.*, 1987) in λEMBL4 (Frischauf *et al.*, 1983) was kindly provided by J. Harmsen. For the isolation of G6PD cDNA a library was made from RNA prepared from *A. niger* N400 mycelium grown under glucose oxidase inducing conditions as described by Witteveen (1990) The cDNA was synthesised (Gubler-Hoffman, 1983) with the BRL cDNA kit as described by the manufacturer. After methylation of internal *Eco*RI sites and addition of *Eco*RI linkers (GGAATTCC) the cDNA was cloned in *Eco*RI digested λZAPII arms and packaged with Gigapack Gold II (Stratagene) extracts.

Lysates were plated and plaque lifts were made on Hybond N$^+$ nylon filters. DNA was simultaneously denatured and covalently fixed to the filters by incubation in a steaming waterbath for 5 min on filterpads soaked in 0.5 M NaOH. The filters were neutralised in 0.5 M Tris-HCl, 1.5 M. NaCl pH 7.5 for 5 min and rubbed clean in 5xSSC, 1% SDS. Filters were prehybridised in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1 M NaCl, 0.5% SDS, 0.1% tetra sodium diphosphate, 10x Denhardt, 100 μg/ml single stranded herring sperm DNA and 10μg/ml polyA for at least three hours at the desired hybridisation temperature. Hybridisation was performed overnight under the same conditions. The filters were washed four times for half an hour at the hybridisation temperature in 5xSSC, 0.5% tetra sodium diphosphate and 1% SDS. For Southern blotting, we incubated the agarose gels in 0.25 M HCl for 30 min and then denatured the DNA by incubating the gels for 30 min in 0.4 M NaOH. The DNA was transferred from the gel to Hybond N$^+$ by capillary blotting in 0.4 M NaOH. The membrane was subsequently washed in 2xSSC and hybridised as described for the plaque lift filters.

Western blot and G6PD detection

Extraction of proteins from mycelium, enzyme assays, electrophoresis, Western blotting and G6PD-specific detection on blots were performed as reported earlier (Wennekes *et al.*, 1993).

Computer analysis

Computer assisted analysis of the DNA sequences was done by means of the GCG software package (Devereux *et al.*, 1984) on a VAX computer.

Results

Cloning of the gsdA gene.

To obtain the G6PD encoding gene from *A. niger*, we screened a genomic library with the 1.0 kb *Eco*RI-*Pst*I fragment from pGW175, encompassing the putative catalytic site of the *Drosophila melanogaster* G6PD enzyme (Hori *et al.*, 1985; Jeffery *et al.*, 1985; Fouts *et al.*, 1988), at 50°C (see Materials and Methods). From this library a set of eight λ clones was isolated. Subsequently the hybridising fragments of the λ inserts were identified by means of restriction analysis and Southern blotting. Seven of these λ clones partially overlapped. Number eight, λ clone 1016, did not overlap with the other seven (fig. 1). With the hybridising fragments two subclones were constructed: pGW252 by cloning the 12 kb *Bam*HI

fragment from λ621 into pEMBL19 and pGW253 by cloning the 7 kb *Eco*RI fragment from λ1016 into pBR328 (fig.1). Detailed analysis of these primary subclones allowed the hybridisation with the *D. melanogaster* probe to be assigned to a 2.8 kb *Sma*I-*Kpn*I fragment in pGW252 and a 1.8 kb *Sal*I fragment in pGW253. The 2.8 kb *Sma*I-*Kpn*I and 1.8 kb *Sal*I fragments were shown to cross hybridise only weakly under stringent conditions. Instead the 1.8 kb *Sal*I fragment hybridised to the 1.2 kb *Kpn*I fragment adjacent to the 2.8 kb *Sma*I-*Kpn*I fragment in pGW252 (data not shown).

The 2.8 kb *Kpn*I-*Sma*I fragment of pGW252 (fig. 1) was used to screen an *A. niger* cDNA library. Several partial cDNA clones were obtained, comprising either the 5'- or 3'-end of the gene. Fortunately some of the 5' and 3' partial cDNA clones proved to overlap by the small internal *Eco*RI fragment (fig. 1). The lack of full length cDNA clones is probably due to incomplete methylation of the internal *Eco*RI sites during the construction of the library.

Cotransformation of the subclones pGW252 and pGW253

To test whether the subclones pGW252 and pGW253 comprised a functional G6PD encoding gene, both were introduced into *A. niger* N593 by cotransformation with the *pyr*A containing vector pGW635

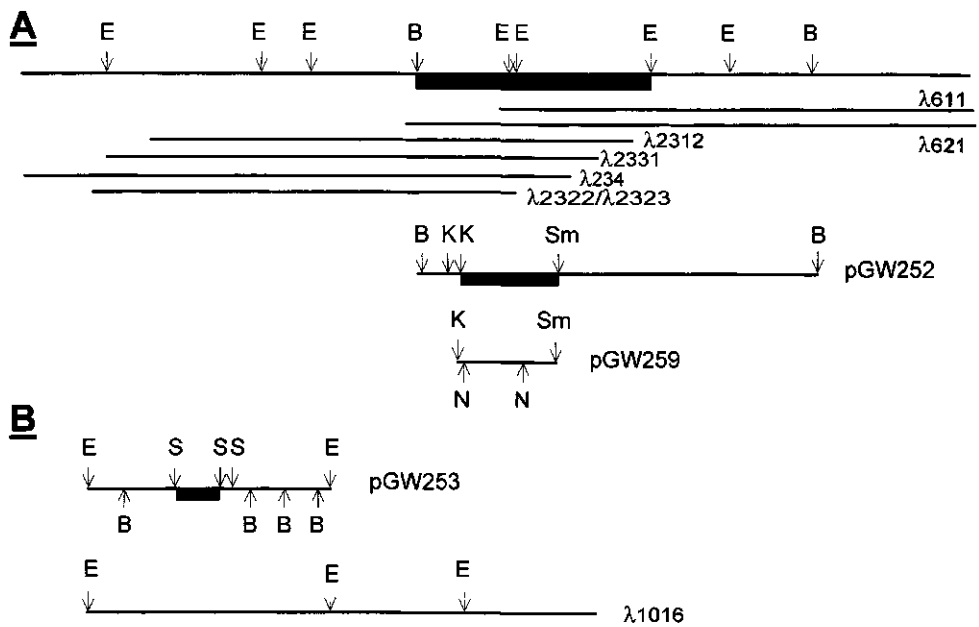


Figure 1. Restriction map of the *A. niger gsd*A gene.

Restriction map of the chromosomal region, that contains the *A. niger gsd*A gene. The seven λ_clones, contributing to this map are represented as thin lines. Restriction sites; B=*Bam*HI, E=*Eco*RI, K=*Kpn*I, N=*Nco*I, S=*Sal*I and Sm=*Sma*I. The regions hybridising with the *D. melanogaster* G6PD probe are indicated by a solid bar. The 12 kb *Bam*HI fragment of λ621 was used to construct pGW252. The restriction map of λ1016, which differs from the other seven clones is also shown. The 7 kb *Eco*RI fragment of λ1016 was subIcloned into pBR328 to give pGW253. As described in the text, both primary subclones pGW252 and pGW253 were used in cotransformation experiments.

(Goosen *et al.*, 1987). As a control 5 µg pGW635 was cotransformed with 20 µg of pUC19 and a transformation efficiency of 264 transformants/µg pGW635 was obtained. This frequency dropped at least tenfold (20 transformants/µg pGW635) when 20 µg pGW252 was used as cotransforming DNA. Only a slight reduction in transformation efficiency was observed when 20 µg pGW253 was used as cotransforming DNA; a frequency of 130 transformants/µg pGW635 was obtained. Twenty-five random-

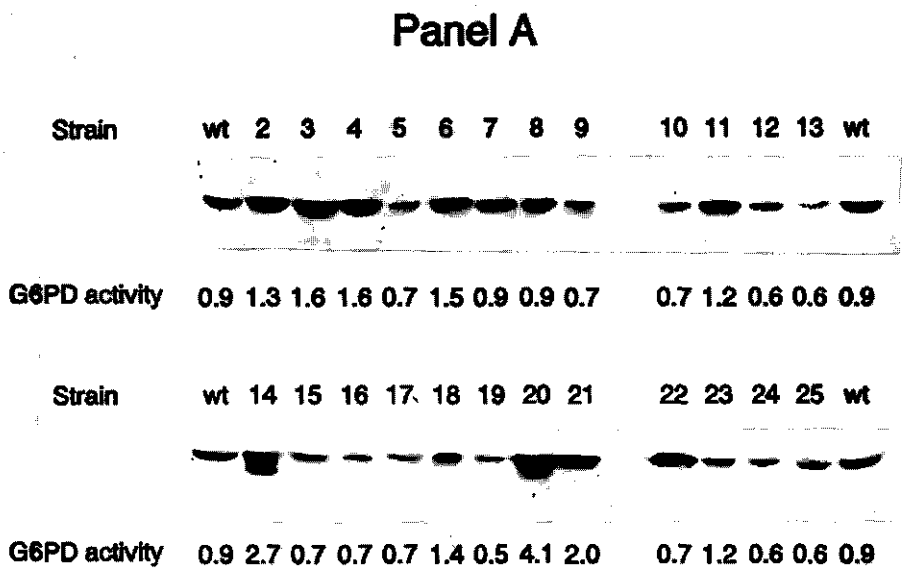
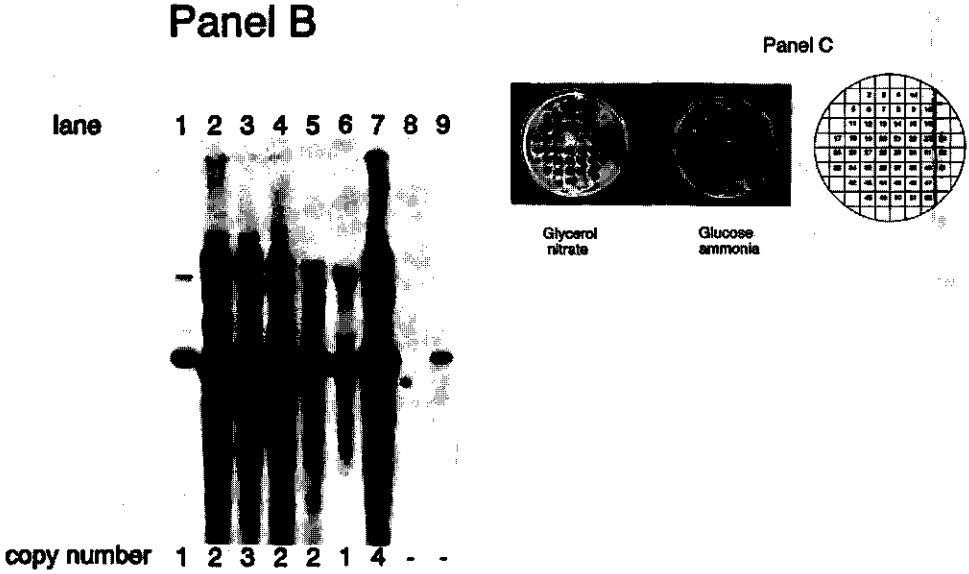


Figure 2. Cotransformation of the subclones pGW252 and pGW253.

Panel A) Protein extracts of 25 transformants of the pGW252 cotransformation experiment were prepared and assayed for G6PD activity. 50 µg protein from each extract was separated on a 10% SDS-PAA gel and electroblotted onto nitrocellulose. The Western blots were stained for G6PD specific bands according to the sandwich procedure (Wennekes *et al.*, 1993). Lanes: 1, 15, 16 and 30 protein extract of a wild-type *A. niger* N402 and lanes 10 and 25 prestained molecular weight marker. The numbers, above the lanes refer to the transformants and the G6PD specific activity is indicated in µmoles NADPH/min/mg protein below the lanes.

Panel B) Chromosomal DNA was digested with *Nco*I separated on an 0.8% agarose gel in 0.5xTBE and transferred to Hybond N$^+$. The blot was probed with the 1.8 kb *Nco*I fragment of pGW259. To estimate the pGW252 copy number, the blot was stripped and rehybridised with the 0.8 kb *Hind*III fragment of the *A. niger trp*C gene (Kos *et al.*, 1985). Lane 1: *A. niger* N402 (wt), lanes 2 to 7: chromosomal DNA of transformants 2, 3, 4, 6, 14, 19, and 20 respectively digested with *Nco*I, lane 8: empty, and lane 9: 1.8 kb *Nco*I probe fragment. The autoradiographs were scanned with a Cybertech CS-1 CCD camera and the ratio of the hybridisation signal of the 1.8 kb *Nco*I fragment and the *trp*C fragment was used to estimate the pGW252 copy number. The estimated copy number is indicated below each lane.

Panel C) Spore suspensions of 25 transformants from both the pGW252 and pGW253 cotransformation experiment were used to inoculate plates. The plates contained minimal medium with either glucose or glycerol as carbon source and either NH_4Cl or $NaNO_3$ as nitrogen source. The plates were incubated at 30°C for 48 hours. The numbers in the inoculation schema, refer to the numbers of the transformants. Numbers 2 to 25 are derived from the pGW252 cotransformation experiment and 26 to 49 are derived from the pGW253 cotransformation. Colonies in positions 50, 51 and 52 are duplos of the transformants in positions 2, 20 and 47 respectively.



ly picked transformants of each cotransformation experiment were analysed for G6PD enzyme activity both in protein extracts and on Western blots.

In the *pyr*A$^+$ transformants, from the pGW252 cotransformation experiment, overexpression of G6PD could be shown in protein extracts. Furthermore in these transformants increased enzyme activity in protein extracts corresponded with enhanced staining of G6PD specific bands on Western blots (fig. 2a).

Chromosomal DNA of potential pGW252 cotransformants 3, 4, 6, 14, 19 and 20 (fig. 2) was analysed in order to establish the presence of the cotransforming DNA. To this end chromosomal DNA was digested with *Nco*I, blotted and probed with the *Nco*I fragment of pGW259 (fig. 1 and 2b). The number of integrated plasmids was estimated by rehybridising the Southernblot with the 0.8 kb *Hind*II fragment of the *A. niger trp*C gene (Kos *et al.*, 1985) and using this signal as a single copy reference. In general the observed increase in G6PD activity corresponded with the number of integrated copies of cotransforming DNA (fig. 2). No increase of G6PD activity was observed in the transformants from the pGW253 cotransformation, although the presence of pGW253 could be demonstrated by probing with the 1.8 kb *Sal*I fragment (data not shown).

The G6PD overexpressing pGW252 cotransformants 3, 4, 6 and 20 (fig. 2c nr. 2-24) exhibit extreme poor growth, especially on medium containing glucose and ammonia. In general the higher the G6PD activity the stronger the observed growth inhibition. Best growth of pGW252 cotransformants, compared to the N402 wild type control, was observed on minimal medium containing either D-glycerol, D-xylose or L-arabinose as carbon source and nitrate as nitrogen source.

The growth characteristics of the transformants from the pGW253 cotransformation (fig. 2c nr. 25-48) were indistinguishable from the N402 wild type control (fig. 2c nr. 4). These results lead to the conclusion that only pGW252 encodes a functional G6PD gene, which hence was named *gsd*A.

Both the internal 1.8 kb *Sal*I and the 3.8 kb *Bam*HI fragments of pGW253 are present in the chromosomal DNA of *A. niger* N402 (not shown). Therefore, λ1016 and its derivative pGW253 are not cloning artefacts.

When *Nco*I digested chromosomal DNA from *A. niger* N402 (fig. 2) is hybridised under stringent

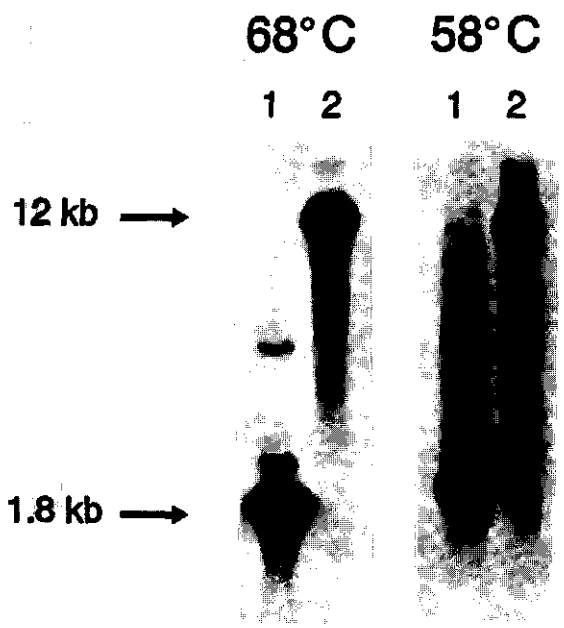


Figure 3. Detection of sequences related to *gsd*A in *A. niger*.
Chromosomal DNA of *A. niger* N402 was digested with *Nco*I and *Bam*HI, separated on an 0.8% agarose gel, blotted and hybridised either at 58°C or 68°C with the 1.8 kb *Nco*I *gsd*A fragment from pGW259. Panel A: Southern blot hybridised at 68°C lane 1: *Bam*HI digest lane 2: *Nco*I digest. Panel B: Blot hybridised at 58°C lane 1: *Bam*HI digest, lane 2 *Nco*I digest. With the probe used, only the 1.8 kb *Nco*I fragment and the 12 kb *Bam*HI fragment should be detected. The additional *Nco*I and *BamH*I fragments can not be derived from the *A. niger gsd*A gene.

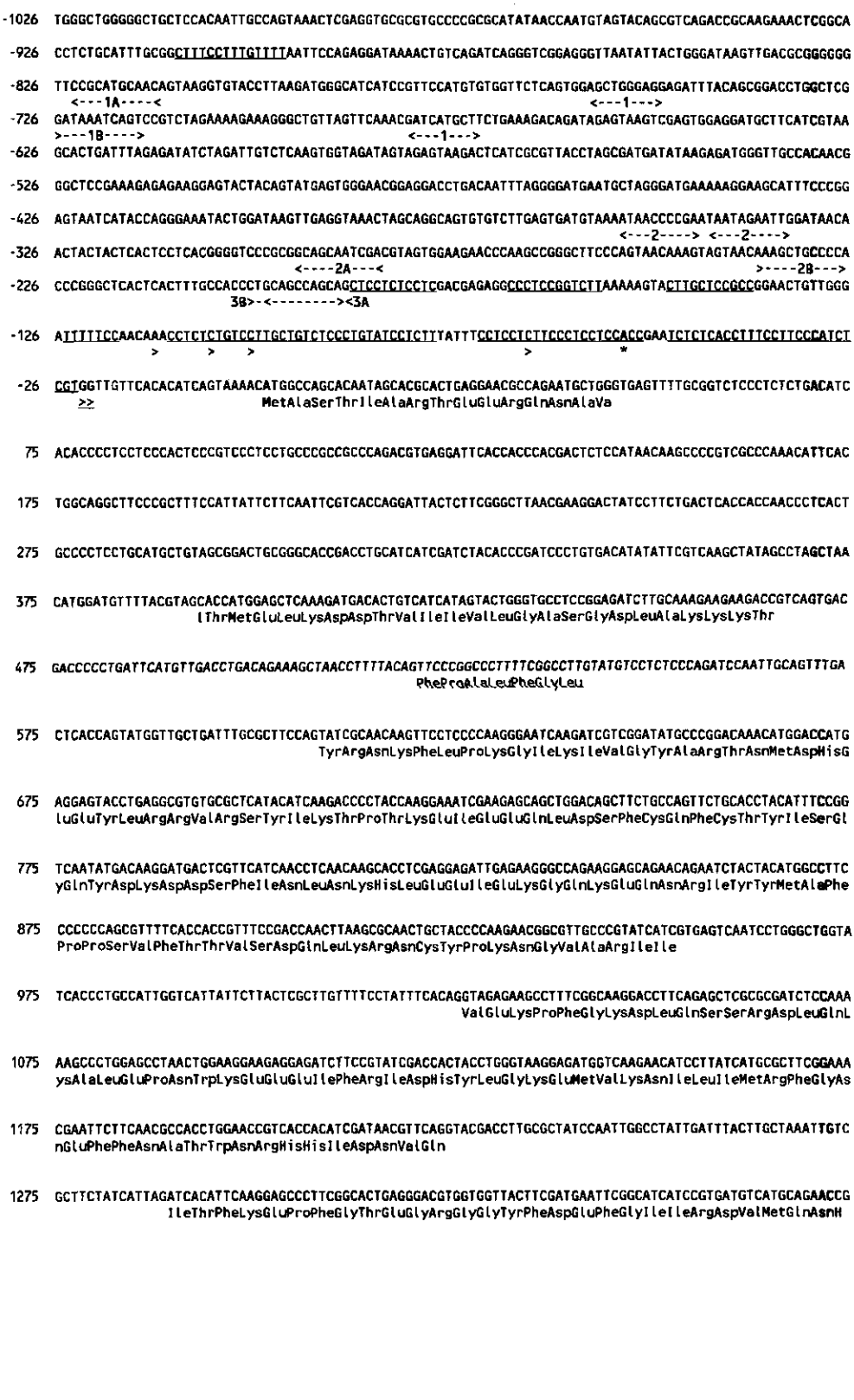
conditions with the *Nco*I fragment of pGW259, not only the expected 1.8 kb *Nco*I band is detected but also two weak bands of higher molecular weight. To investigate this further, chromosomal DNA of *A. niger* N402 was digested with *Nco*I or *Bam*HI, transferred to a nylon membrane and hybridised at 58°C with the *Nco*I fragment from the *gsd*A gene. In the *Nco*I digest again three bands hybridised, but the signal of the larger *Nco*I fragments became stronger.
Also in the *Bam*HI digest one weak additional band of higher molecular weight is visible (fig. 3). Since the two additional *Nco*I bands were observed in two separate experiments, it is unlikely that these bands are the result of a partial digestion of the chromosomal DNA. Neither the two *Nco*I bands, nor the weak *Bam*HI band can be attributed to the *gsd*A gene, which raises the possibility of a second G6PD encoding gene in *A. niger*. These cross hybridising bands are not related to λ1016, which is another indication that this clone does not contain a G6PD encoding gene.

Nucleotide sequence of the *gsd*A gene

The nucleotide sequences of the genomic and cDNA clones were determined according to a strategy in which both strands of partially overlapping subclones were analysed. The results are summarised in figure 4.

Introns

The introns in the *A. niger gsd*A gene were localised by alignment of the genomic and cDNA sequences. Nine introns interrupt the coding region, but none are found in the 5' and 3' non-translated sequences. Since no sequences resembling splice signals (Gurr, 1987) occur in the *gsd*A gene between the 5' end



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1375  TACGTTCAAAGTCACGCTCGACATCTCCGACATGATGCTGATAAAAATCTCTCCTAGACCTTCTCCAGGTGTTGACGCTGCTCGCTATGGAGCGCCCCAT
                                                                    isLeuLeuGlnValLeuThrLeuLeuAlaMetGluArgProIl

1475  TTCCTTCTCCGCCGAGGACATCCGTGACGAGAAGGTACAGTGTGCGCTTGACTATTGGTTGTGCTGGGTTACTGACACTTAACCAGGTTCGTGTCCTCCG
      eSerPheSerAlaGluAspIleArgAspGluLys                                                          ValArgValLeuAr

1575  TGCGATGGACGCCATTGAGCCCAAGAACGTCATTATTGGCCAGTACGGAAAGTCTCTGGATGGCAGCAAGCCCGCCTACAAGGAGGACGAGACCGTTCCC
      gAlaMetAspAlaIleGluProLysAsnValIleIleGlyGlnTyrGlyLysSerLeuAspGlySerLysProAlaTyrLysGluAspGluThrValPro

1675  CAGGATTCCCGCTGCCCCACCTTCTGCGCTATGGTCGCCTACATCAAGAACGAGAGGTGGGACGGTGTTCCTTTCATCATGAAGGCTGGCAAGGGTATGT
      GlnAspSerArgCysProThrPheCysAlaMetValAlaTyrIleLysAsnGluArgTrpAspGlyValProPheIleMetLysAlaGlyLysA

1775  ACCTCTTTCCAAGCGATCATAGCACCGATTGGTATACTAATAATTCGCAGCCTTGAACGAGCAGAAGACCGAGATCCGTATCCAGTTCCGTGACGTTACC
                                                                    laLeuAsnGluGlnLysThrGluIleArgIleGlnPheArgAspValThr

1875  TCCGGAATTTTCAAGGACATCCCTCGCAACGAGCTCGTTATCCGCGTCCAGCCCAACGAGTCCGTGTACATCAAGATGAACTCCAAGCTGCCTGGCCTGT
      SerGlyIlePheLysAspIleProArgAsnGluLeuValIleArgValGlnProAsnGluSerValTyrIleLysMetAsnSerLysLeuProGlyLeuS

1975  CCATGCAGACGGTTGTGACTGAGCTCGACCTCACCTACCGCCGCCGCTTCTCCGACCTCAAGATCCCCGAAGCCTACGAGTCTCTGATCCTGGATGCTCT
      erMetGlnThrValValThrGluLeuAspLeuThrTyrArgArgArgPheSerAspLeuLysIleProGluAlaTyrGluSerLeuIleLeuAspAlaLe

2075  GAAGGGCGACCACTCCAACTTCGTCCGTGACGATGAGCTGGATGCCAGCTGGAGGATCTTCACCCCTCTCCTGCACTACCTGGATGACAACAAGGAGATC
      uLysGlyAspHisSerAsnPheValArgAspAspGluLeuAspAlaSerTrpArgIlePheThrProLeuLeuHisTyrLeuAspAspAsnLysGluIle

2175  ATCCCCATGGAATACCCCTACGGTACGTGCACTTCTTGCAATTTGTCTAAATCGCTTACATACTGACCAACGCGCAGGCTCCCGCGGACCCGCCGTCCTT
      IleProMetGluTyrProTyrG                                                                      lySerArgGlyProAlaValLeu

2275  GATGACTTCACCGCGTCCTTCGGCTACAAGTTCAGCGATGCTGCTGGCTACCAGTGGCCCTTGACTTCCACCCCAACCGTCTGTAAATAAGGGCGGTCGG
      AspAspPheThrAlaSerPheGlyTyrLysPheSerAspAlaAlaGlyTyrGlnTrpProLeuThrSerThrProThrValCysLysEND

2375  CAGGTTATGACGGATGAGGATGAAAAAAAAAATTATTGCCAAAAAAAGGCTAAAAAAAAAGATGTTAATGCGATTGATTTTCGGTCGAGAATCATGGTATGAC

2475  GGGGCATCTGGGATGATATGACAGAAATGAAGCACTCGGGACTATTTATCGGTCGGCTGGGCAATAACTGGAGTTATCTATTCGCAACCCCTTTTTAGAA

2575  ACGAATGCAGAGAACGTAACGAACCACCCCCGGTCGGTTGGGACCGCCGCTGTCTTCACCGGAAGCTGGAAGTAGTCGCGTGACCGTA  2662
                                *AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 4. Nucleotide and deduced amino acid sequence of the *A.niger gsd*A gene.

The positions of the introns and the 1530 bp open reading frame were deduced by alignment with the cDNA sequence. The TATA-box like sequences, initiation and stop codons are indicated in bold print. The transcription start sites, mapped by a S1 nuclease protection assay (see fig. 6), are marked by > signs and those mapped by primer extension by ≥ signs. The putative CT boxes are underlined. The direct repeats (1 and 2) and the inverted repeats (1a-1b, 2a-2b and 3a-3b) are marked by dashed arrows. The start of the longest cDNA is marked by an asterisk and the beginning of the polyA track in the cDNA is indicated by an asterisk followed by As. In the 3' non-translated trailer the $CA^T/_C{}^A/_GG$ homology and AATAAA-like putative polyadenylation signals are doubly underlined.

of the cDNA and the transcription start (fig. 4), the presence of an additional intron in the 5' non-coding region is unlikely.

The first intron in the *gsd*A gene is 348 bp long, which is exceptionally large for an intron from filamentous fungi (Unkles, 1992). The other eight introns are 51 to 72 bp long, a length more frequently encountered in filamentous fungi (Gurr *et al.*, 1987; Unkles, 1992).

With the exception of intron number 7, all deviations from the fungal intron-exon boundary consen-

sus (Rambosek and Leach, 1987; Gurr *et al.*, 1987) are single base substitutions. The aberrant donor splice site on intron number 7 carries an additional A, instead of a substitution. The position of intron 7 is, with respect to the coding region, equivalent to the position of intron 8 in the human G6PD gene (Chen *et al.*, 1991). Apart from intron 7 no conservation of the position of the introns relative to the coding region is found in comparison with the human or Drosophila G6PD gene (Fouts *et al.*, 1988: Chen *et al.*, 1991).

Deduced amino acid sequence

In the *A. niger gsd*A cDNA two potential initiation codons are present; one in position +1 and one in position +397 (fig. 4). Both ATGs in the *gsd*A cDNA share the important features with the GCC($^{A}/_{G}$)CCAUGG consensus (Kozak, 1986). The sequence context of the second ATG in the *A. niger gsd*A cDNA is identical to the sequence ACCATGG, which proved to be optimal for initiation of translation by eukaryotic ribosomes (Kozak, 1986).
From the 5' proximal ATG (pos.+1, fig. 4) in the cDNA to the stopcodon TAA (pos. 2362), the *A. niger gsd*A gene encodes a protein of 510 AA with a deduced molecular weight (M_w) of 58.951 kD. Using the second ATG (pos. +397) a protein of 495 AA and a M_w of 57.232 kD is encoded. This 1.6 kD difference in molecular weight matches the difference observed on SDS polyacrylamide gels between the two G6PD monomers (Wennekes *et al.*, 1993).
The deduced amino acid sequence contains a sequence of 11 amino acid residues (pos. 1112-1145), that is identical to the domain in yeast and human G6PD comprising the reactive lysine (Jeffery *et al.*, 1985: Camardella *et al.*, 1988). The *gsd*A open reading frame also encodes a 30 amino acid residues long sequence (pos. 1893-1983), that is for 60% identical to the putative $NADP^+$ binding site in human G6PD (Hirono *et al.*, 1989).

3' end non-translated sequences

Two sequences implicated in polyadenylation have been described for mammalian protein encoding genes: the AAT$^{T}/_{A}$AA consensus and the CA$^{T}/_{C}{}^{A}/_{G}$G consensus (Proudfoot and Brownlee, 1976: Benoist *et al.*, 1980). Especially the AAT$^{T}/_{A}$AA consensus is strongly conserved and only very few shortened versions have been described (Berget, 1984). Both the CA$^{T}/_{C}{}^{A}/_{G}$G and AAT$^{T}/_{A}$AA consensus are thought to be recognised by the RNA moiety of mammalian U4 small ribonucleoproteins, which therefore are considered to be involved in polyadenylation (Berget, 1984).

The poly A track of the *gsd*A cDNA starts at position 2601, 239 bp downstream of the stopcodon (pos. 2362). No sequences fitting the highly conserved AAT$^{T}/_{A}$AA consensus of mammalian polyadenylation sites are encountered within this region. This lack of canonical polyadenylation signals is reported to be common among genes of filamentous fungi (Gurr *et al.*, 1987).

Four sequences, resembling the AAT$^{T}/_{A}$AA consensus are encountered: AATAAG (pos. 2360), AATTAT (pos. 2404), AATGAA (pos. 2500) and AATAAC (pos. 2537). In all four cases the poly A addition site (pos. 2601) is further downstream than the 1-26 bp reported for mammalian polyadenylation sites (Berget, 1984). A single copy of a sequence CATGGT (pos. 2364), fitting the consensus CA$^{T}/_{C}{}^{A}/_{G}$G, is observed in the 3' non-coding region of the *A. niger gsd*A gene. This sequence also resembles the consensus sequence CATGGTTCT found downstream of the AATAAA motif in a number of fungal genes (Gurr *et al.*, 1987). Apart from the aforementioned homologies three short stretches of A's are the only remarkable features in the non-translated trailer sequences from the *A. niger gsd*A gene.

Figure 5. Intron-exon boundary sequences in the *A niger gsd*A gene.

Intron	5'	lariat	3'	Length of intron
1	GTGAGT	AGCTAACA	CAG	348
2	GT**C**AGT	AGCTAACC	CAG	55
3	GTATGT	TGCTGA**T**T	CAG	65
4	GTGAGT	T**T**CT**T**ACT	CAG	71
5	GTACG**A**	TACT**T**G**C**T	TAG	65
6	GTACGT	TGCTGA**T**A	TAG	58
7	GTAC**A**GT	TACTGACA	CAG	52
8	GTATGT	TACTAA**T**A	CAG	56
9	GTACGT	TACTGACC	CAG	55
Consensus	**GT$^{G}/_{A}$NGT**	**N$^{G}/_{A}$CT$^{G}/_{A}$ACN**	**$^{C}/_{T}$AG**	

The nucleotides deviating from the consensus (Rambosek and Leach, 1979) are underlined in bold print.

Transcription start and 5' non-transcribed sequences

The transcription start points (*tsp*) from the *A. niger gsd*A gene was determined by a S1 nuclease protection assay on 50 µg total RNA extracted from mycelium grown on minimal medium containing glucose and nitrate. In figure 6 two strong bands and two bands of lesser intensity are visible, mapping the two major *tsp*s to C's in positions -107 and -101 and the minor *tsp*s to a C in position -67 and an A in position -114 of the *gsd*A sequence (fig. 4). All *tsp*s are located within a C+T rich sequence as is often observed in fungal genes (Gurr *et al.*, 1987; Punt *et al.*, 1990). There is a TATA box like sequence TATTT (fig. 4 pos. -78), immediately upstream of a C+T rich track. TATA-like boxes preceding similar C+T rich stretches have also been reported in several *A. nidulans* and *N. crassa* genes (Ballance, 1986; Punt *et al.*, 1988). Given its position downstream of the two major transcription start points, it is very unlikely that this TATA-box is functional under the conditions tested. Other TATA-like sequences (-550, -854 and -969) reside much farther upstream and are therefore probably also not functional. Sequences clearly fitting the CAAT-box consensus GCCAAT (Benoist *et al.*, 1980) are absent from the *gsd*A promoter region.

Primer extension, performed with the thermostable reverse transcriptase TET-z, mapped the transcription starts in *A. niger* to two G's (pos. -23 and -22 fig. 4) just downstream of a C+T rich stretch. Since the sequence of the longest cDNA clone starts 20 bp upstream (pos. -55) of these two G's, just downstream of a C+T rich stretch, this is believed to be an artefact of the TET-z extension.
Computer analysis of the sequence 1000 bp upstream of the initiation codon reveals the presence of several direct and inverted repeats (fig. 4) in the *A. niger gsd*A promoter.

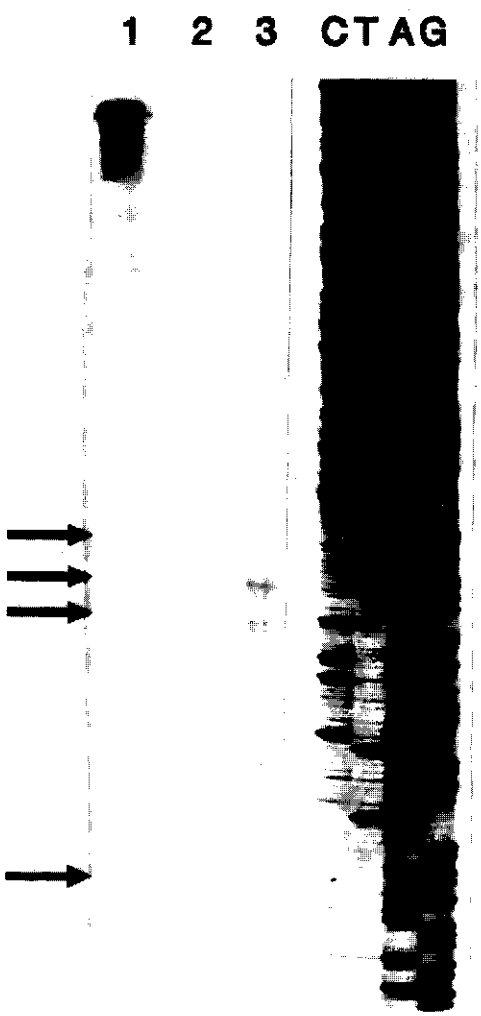


Figure 6. Mapping of transcription start points.

S1 protection assay on 50 µg total RNA. lane 1: S1 probe, lane 2: S1 probe hybridised to 50 µg total *A. niger* N402 RNA and digested with S1 nuclease, lane 3: S1 probe hybridised to 50 µg total yeast RNA and treated with S1 nuclease and lane 4 to 7 sequence ladder. In all experiments the sequence ladder and the S1 probes were prepared with oligonucleotide 5'-CCTCAAGTGCGTGCTATTGTGCTGGCC-3' and plasmid pGW255 as template.

Attempts to construct a *gsd*A disruption strain by a two step gene replacement strategy

The poor growth of G6PD overproducing transformants demonstrated the importance for proper control of G6PD expression. To explore the role of *gsd*A in the control of the NADPH/NADP$^+$ ratio, we attempted to construct a *gsd*A null mutant. Because initial direct gene replacement experiments failed to yield the desired mutant, it could not be excluded that disruption of the *gsd*A gene would be lethal. Therefore a two step gene replacement strategy (Miller *et al.*, 1985) was adopted. In this two step gene disruption strategy the pGW866 vector (fig. 7) with a mutant *gsd*A allele and the *Claviceps purpurea pyr*4 gene (Smit *et al.*, 1992), has to be integrated at the *gsd*A locus (fig. 7). The mutant allele was constructed by insertion of a hygromycin B resistance gene (Punt *et al.*, 1989) into the *gsd*A coding region.

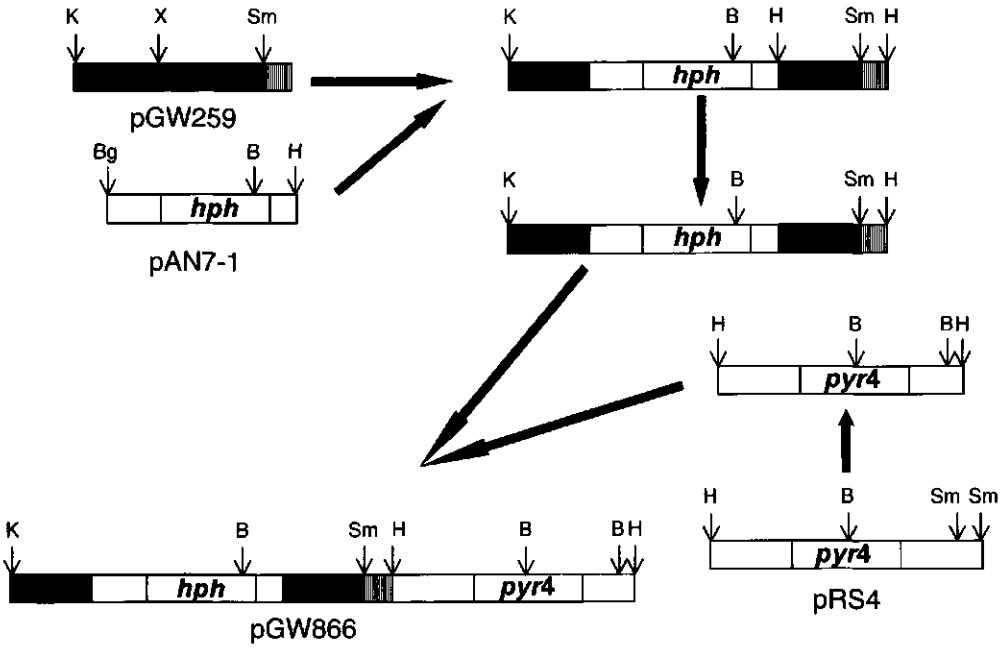


Figure 7. Construction of *A. niger gsd*A gene disruption plasmid pGW866.

A mutant *gsd*A allele was generated by insertion of the hygromycin B resistance cassette from pAN7-1 (Punt *et al.*, 1987) into the coding region of the *gsd*A gene. The cassette is a *Hind*III-*Bgl*II fragment containing the bacterial hygromycin B resistance gene fused to the *A. nidulans gpd*A promoter and the *trp*C terminater. To create the plasmid pGW860, the *Hind*III-*Bgl*II fragment from pAN7-1 was blunt ligated into the blunt ended *Xho*I site of pGW259. The recreated *Hind*III site was eliminated from pGW860 by partial digestion with *Hind*III, filling in the ends followed by selfligation. This plasmid was named pGW862. The plasmid pRS4 (Smit *et al.*, 1992) containing the *Claviceps purpurea pyr*4 gene, was digested with *Sma*I, gel purified and selfligated in the presence of a 10 bp *Hind*III linker. The *pyr*4 gene was subsequently cloned into the remaining *Hind*III site of pGW862 to give the *gsd*A gene disruption construct pGW866. Restriction sites B=*Bam*HI, Bg=*Bgl*II, E=*Eco*RI, H=*Hind*III, K=*Kpn*I, Sm=*Sma*I and X=*Xho*I. Since both *pyr*4 and the hygromycin B resistance cassette contain *Bam*HI sites, integration of pGW866 at the *gsd*A locus adds these sites to the wild type 12 kb *gsd*A *Bam*HI fragment. Therefore, homologous integration of pGW866 can be detected in Southern blots of genomic DNA from transformants by the loss of the wild type 12 kb *Bam*HI *gsd*A fragment.

Subsequently a *gsd*A gene disruption can be selected by screening for hygromycin B resistant *pyr*4$^-$ revertants.

pGW866 was used to transform *A. niger* N755 to uracil prototrophy. More than 95% of the *pyr*4$^+$ transformants proved to be hygromycin resistant as well. In a transformant carrying pGW866 integrated at the *gsd*A locus, loss of the *pyr*4 marker occurs most likely by a recombination event between the two *gsd*A alleles.

Therefore, such a transformant should produce 5-fluoroorotic acid resistant sectors at a higher frequency than transformants carrying a heterologously integrated pGW866 plasmid. For in a transformant with a heterologous integration of pGW866, *pyr*4$^-$ revertants should only occur by mutation.

Single spore isolates of twenty primary transformants were stabbed onto CM supplemented with uri-

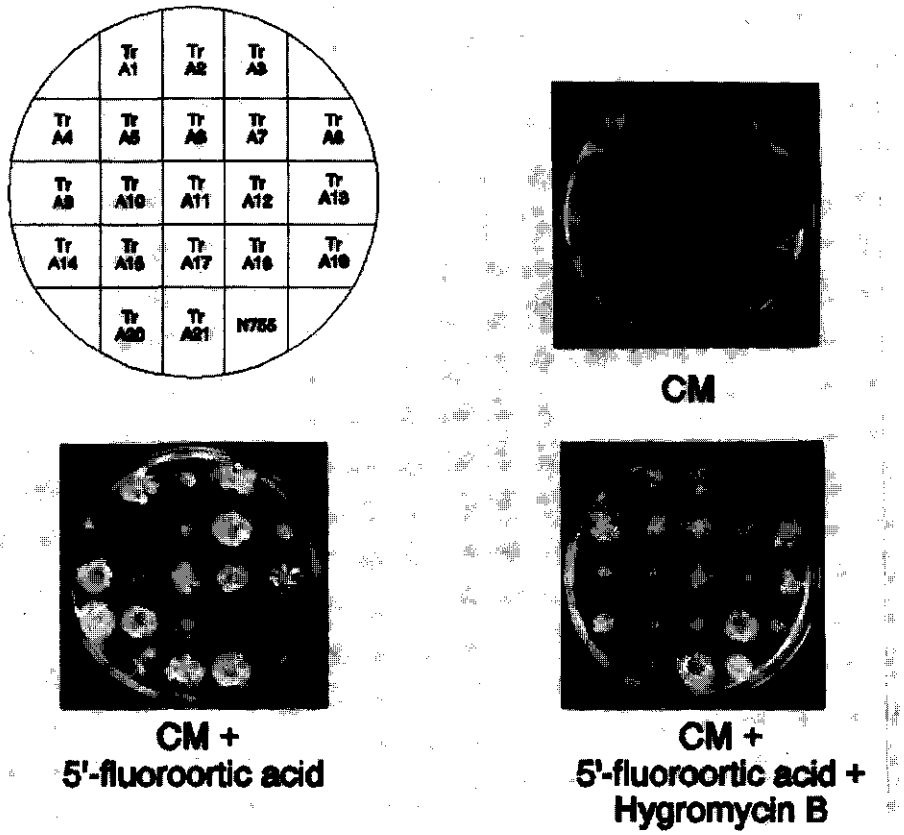
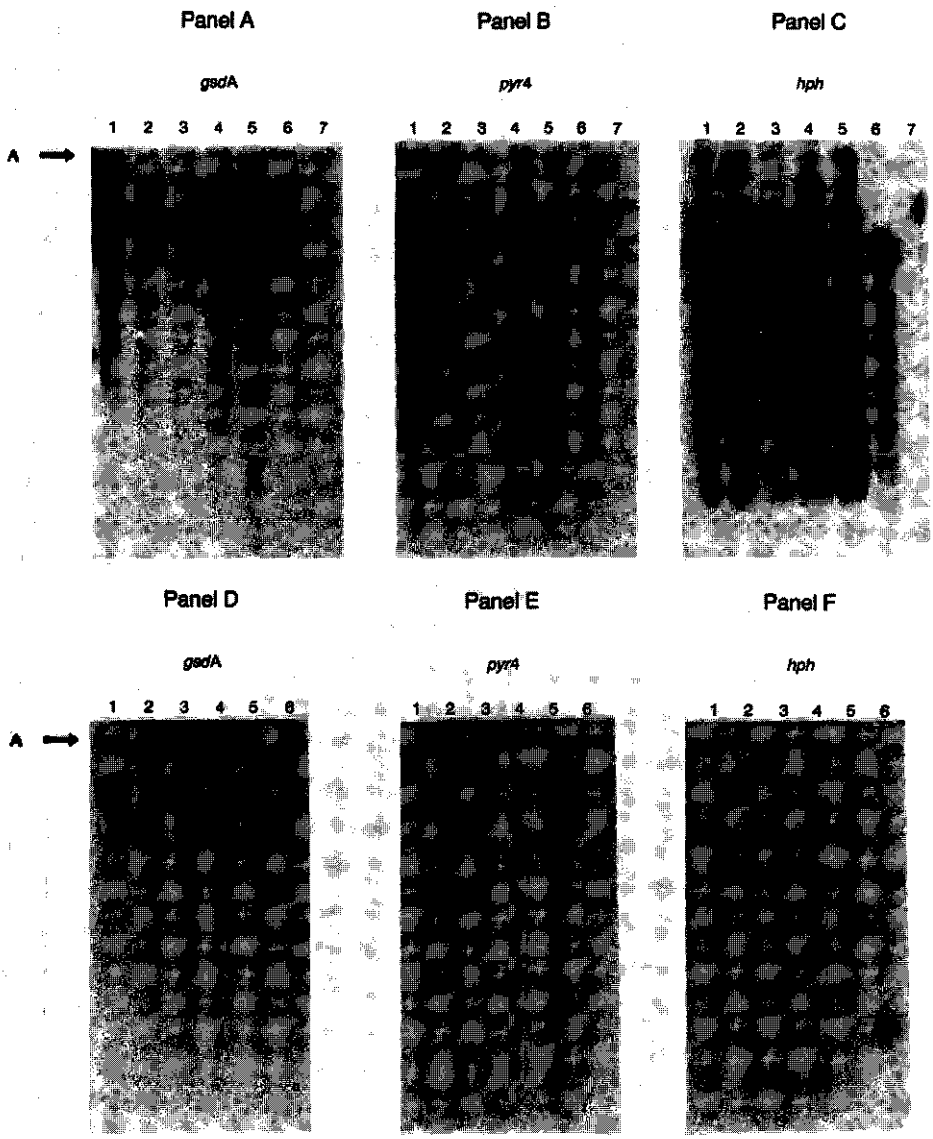


Figure 8. Selection and Southern analysis of hygromycine B resistant *pyr*4 revertants. Conidiospore suspensons of twenty-one pGW866 transformants were spotted on CM plates, containing either 5-fluoroorotic acid and hygromycin B or 5-fluoroorotic acid alone. The CM was supplemented with uridine, nicotinamide and methionine. The plates were incubated for 48 hours at 30°C. Some transformants developed hygromycin B and 5-fluoroorotic acid resistant sectors at a high frequency. 2) Identical Southern blots of *Bam*HI digested chromosomal DNA from six transformants and six hygromycin B resistant *pyr4*⁻ revertants were hybridised with either the 1.8 kb *Nco*I *gsd*A fragment (panel A), the 1 kb *Sst*I-*Eco*RI *C. purpurea pyr*4 fragment of pGW862 (panel B) and the *hph* fragment from pAN7-1 (panel C). (panel A to C: lanes 1 to 6 *A. niger* N755 transformants and lane 7, *A. niger* N402) Integration of pGW866 at the *gsd*A locus would result in the loss of the parental 12 kb *Bam*HI *gsd*A band (Fig. 7). All six transformants retain this parental *gsd*A *Bam*HI band, but contain the *pyr*4 gene and the hygromycin B resistance gene. In all hygromycin B and 5-fluoroorotic acid resistant revertants of the pGW866 *pyr4*⁺ transformants, the parental 12 kb *Bam*HI *gsd*A band hybridises (panel D lanes 1 to 6) indicated by an arrow) which proves that the *gsd*A gene is not disrupted. The *pyr4*⁻ revertants 1, 4 and 6 lost all copies of the *pyr*4 gene (panel E lanes 1 to 6), but retained the hygromycin B resistance cassette (panel F lanes 1 to 6).



dine and methionine containing either hygromycin B and 5-fluoroorotic acid or 5-fluoroorotic acid alone. Methionine was added to the selection plates, because *S. cerevisiae* G6PD null mutants have a still unexplained requirement for organic sulphur compounds (Thomas *et al.*, 1992). About 50% of the transformants exhibited the expected high frequency of 5-fluoroorotic acid resistant sectors and these sectors were transferred to CM supplemented with methionine and uridine. Southern analysis (fig. 8) of these transformants and their 5-fluoroorotic acid and hygromycin resistant descendants showed that all of them had an intact *gsd*A gene. Further analysis confirmed that some of them had completely lost the *pyr*4 marker, while retaining the hygromycin B resistance gene. Apparently the *pyr*4 marker can excise

itself from the chromosome, without the aid of recombination between the wild type and mutant *gsd*A alleles. This property makes pGW866 unsuitable for constructing a *gsd*A null mutant.

Discussion

By screening an *A. niger* genomic library with a *D. melanogaster* G6PD probe two distinct types of λ clones were isolated. pGW252, a subclone derived from the first type of λ clones, caused an increase in G6PD activity when introduced into *A. niger*. Overexpression of G6PD in cotransformants *per se* does not prove that pGW252 encodes a G6PD enzyme, because it does not exclude the possibility that the cotransformed DNA merely encodes a G6PD regulator protein. However, the homology to other eukaryotic G6PDs of the protein encoded by the cross hybridising fragments of pGW252, strongly argues that this plasmid contains a G6PD encoding gene. Furthermore, the overexpression showed that the cloned G6PD gene (*gsd*A) is functional.

The isolation of two distinct types of λ clones appears to be consistent with the observation that pure G6PD enzyme preparations from *A. niger* contain two different monomers (Wennekes *et al.*, 1993). However the second type of clone, λ1016, cross hybridises only weakly to the *gsd*A gene. Moreover the λ1016 subclone pGW253 fails to increase G6PD activity, when introduced into *A. niger*. Therefore λ1016 probably does not contain a functional G6PD encoding gene.

The *A. niger gsd*A gene has many features typical for genes from filamentous fungi; multiple transcription start sites, introns, but no canonical TATA box, CCAAT box or polyadenylation sites. Nonetheless, both the large number of introns and the length of the first intron are exceptional for fungal genes (Gurr *et al.*, 1987; Rambosek and Leach, 1987; Unkles, 1992).

In the *gsd*A gene all transcription start sites map within a long C+T rich stretch, which is in agreement with the proposed role of CT boxes in fungal promoters: CT boxes are supposed to direct the transcription machinery to the transcription start point (Punt *et al.*, 1990). The discrepancy between the transcription starts mapped by primer extension and S1 protection, points to the inability of TET-z reverse transcriptase to read through C+T rich sequences. In this respect it is interesting to note, that the cDNA sequence also stops downstream of a long C+T rich sequence; it is possible that the inability to read through C+T rich sequences is a general property of reverse transcriptases. In control experiments the primer extension with TET-z also failed to map the transcription start of the *A. nidulans gpd*A gene to the previously published position (Punt *et al.*, 1988). The latter finding indicates TET-z cannot be used as an alternative for reverse transcriptase in transcription start mapping.

If genomic DNA from *A. niger* is probed with a fragment, which constitutes most of the *gsd*A coding region, sequences are detected that could represent a second G6PD encoding gene. Although the potential second gene cross hybridises under stringent conditions, all cDNA clones isolated are clearly derived from the *gsd*A gene. This indicates that this potential second gene is not constitutively expressed and therefore cannot be the explanation for the two monomers. Moreover in pGW252 cotransformants both monomeric forms are overexpressed, which also suggests that they are both derived from the *gsd*A gene.

In the related fungus *Aspergillus oryzae* also two G6PD isoforms are present in glucose grown mycelium and a third specific G6PD isoform is induced during growth on ribose (Cebrian-Perez *et al.*, 1990). It is therefore conceivable that the cross hybridising sequences represent a second G6PD encoding gene, induced only under specific conditions.

If we assume that both G6PD monomers are encoded by the *gsd*A gene, then the cause of the difference in their molecular weight needs to be explained.

The presence of two G6PD monomers in pure enzyme preparations of *A. niger* (Wennekes *et al.*, 1993) could be explained by alternative use of both putative initiation codons in the *gsd*A cDNA. It was shown that downstream ATGs are used, if the 5'-proximal ATG poorly fits the GCC(A/G)CCAUGG consensus (Kozak, 1986). Analysis of the effect of single base substitutions around the ATG of rat preproinsuline on the initiation of protein synthesis, showed that the nucleotides in the positions -3 and +4 (the A in AUG being +1) of the consensus are the major determinants for efficient initiation codon recognition (Kozak, 1986). Since both potential initiation codons share these two important features with the consensus, it is doubtful whether these two monomers arise by differential translation initiation. Additionally this hypothesis fails to explain the inconsistency between the deduced and determined molecular weight of the *A. niger* G6PD.

The deduced molecular weight of the protein encoded by the *gsd*A gene is in the same range as other eukaryotic G6PDs, but differs from the molecular weight of both *A. niger* G6PD monomers determined on SDS-polyacrylamide gels (Wennekes *et al.*, 1993). It is unlikely that this difference is caused by an electrophoresis artefact, for the molecular weight of the *S. cerevisiae* G6PD (59 kD) and the two *A. nidulans* monomers (60 and 57 kD), estimated from the same gel, are compatible with the deduced molecular weight of respectively 57.4 kD (Thomas *et al.*, 1991) and 58,6 kD (van den Broek *et al.* unpublished). Hence the most plausible explanation for both the discrepancy in molecular weight and the two G6PD monomers is either proteolytic degradation during isolation or post translational processing.
The extremly poor growth of the transformant carrying four copies of the *gsd*A gene suggests that additional copies would result in the death of the fungus.
This toxicity of G6PD overexpression would also explain the dramatic drop in transformation efficiency, when pGW252 was introduced into *A. niger*. In contrast to the *gsd*A gene, multiple copies of the glucoamylase gene (*gla*A) and acetamidase gene (*amd*S) can be introduced into *A. niger* and expressed without adverse effects on the fungus (Kelly and Hynes, 1985; Verdoes *et al.*, 1993).
On the other hand, 10-50 copies of the *amd*S gene cause titration of regulatory factors in *A. niger*, which influences the expression of coregulated genes and leads to reduced growth on ω-amino acids (Kelly and Hynes, 1985).
As opposed to the behaviour of the *A. niger gsd*A gene no increase in G6PD activity is observed in transformants of *S. cerevisiae*, not even when the G6PD gene (*ZWF*1) is present on a plasmid attaining 100-500 copies per cell (Nogae and Johnstone, 1990). This suggests that regulation of G6PD expression is different in *A. niger* and *S. cerevisiae*. The absence of increased G6PD activity in *ZWF*1 multicopy transformants, contradicts the conclusion by Nogae and Johnstone (1990) that G6PD expression in *S. cerevisiae* is not regulated.

In general pGW252 cotransformants grow better on carbon- and nitrogen sources requiring reduction by NADPH, which is the opposite of the behaviour of *N. crassa* G6PD mutants (Nisikawa and Kuwana, 1983) and *A. nidulans* pentose phosphate pathway mutants (Hankinson, 1974).

Generally mutants provide important information on the physiological role and regulation of a particular gene. G6PD mutants of *D. melanogaster* (Gvozdev *et al.*, 1977), *S. cerevisiae* (Thomas *et al.*, 1991) and *E. coli* (Fraenkel, 1968) were isolated and did not have phenotypes that can be explained with lack of NADPH. On the other hand G6PD mutants in humans (Beutler, 1989) and *N. crassa* (Scott and Mahoney, 1976) have phenotypes, that are clearly the result of decreased NADPH production. Attempts to make an *A. niger gsd*A null mutant failed, because we were unable to find a transformant with the disruption construct integrated at the *gsd*A locus. This is surprising since we deliberately introduced no other *A. niger* sequences than *gsd*A into the disruption construct in order to optimise the frequency of integration at the *gsd*A locus. The disruption construct does not appear to have integrated at random, because the hybridisation patterns of the transformants are very alike.

In *Aspergillus* transformations homologous integration of transforming DNA, is frequently observed and the feasibility of gene disruptions in filamentous fungi has also been demonstrated (Miller *et al.*, 1985). However the frequency of homologous integration of gene disruption constructs varies considerably. The mutant SpoC1-C allele, constructed by substitution of an internal fragment with the *A. nidulans trp*C gene, resulted in several homologous integration events among 32 transformants analysed (Miller *et al.*, 1885). On the other hand, it took 6150 *A. niger* transformants to find 8 *trp*C null mutants after a one step gene disruption experiment (Goosen *et al.*, 1989).

To add to our misfortune even when pGW866 is heterologously integrated, the *pyr*4 marker is able to excise itself from the chromosomal DNA at a high frequency. Since the sequence of the pRS4 plasmid has not been elucidated, we can only speculate about an explanation for this phenomenon. It could be that both ends of the *pyr*4 insert are homologous, but still the frequency of excision is remarkably high.

Since we are unable to construct a *gsd*A null mutant, the physiological role of the gene must be deduced in an other way. A good alternative would be to study *gsd*A gene expression by Northern blotting under different physiological conditions. In combination with a promoter deletion study, also the elements responsible for changes in transcription level could be identified. It would be interesting to see whether the direct and inverted repeats in the *gsd*A promoter represent promoter elements.

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Chapter 4 Isolation and characterisation of the Glucose-6-phosphate dehydrogenase gene (*gsd*A) from *Aspergillus nidulans*

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Summary

The gene encoding glucose-6-phosphate dehydrogenase (*gsd*A) was isolated from *Aspergillus nidulans* and characterised. The functionality of the cloned gene was demonstrated by overproduction of G6PD upon introduction into *A. nidulans*.

At the amino acid sequence level, there is 92% identity between the *A. nidulans gsd*A gene product and the G6PD from *Aspergillus niger*. Comparison of the deduced amino acid sequences of the two *Aspergillus gsd*A genes with other eukaryotic G6PDs, allows functionally important domains to be identified. Also the primary structure of the genes, including the positions of the introns have been strongly conserved between the two fungi. The strong sequence conservation in the coding region contrasts with the low overall homology of the introns and of the 5' and 3' proximal regions in the two *gsd*A genes. Only four short stretches of highly conserved sequences are encountered in the putative *gsd*A promoter regions and two in the 3' proximal regions. Nevertheless, the *A. nidulans gsd*A gene is expressed when introduced into *A. niger*.

Introduction

Several observations suggest that glucose-6-phosphate dehydrogenase (G6PD: ß-D-glucose-6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49) and 6-phospho-gluconate dehydrogenase (6PGD: 6-phosphogluconate: $NADP^+$ 2-oxidoreductase, EC 1.1.1.44), the two dehydrogenases from the pentose phosphate pathway, are the primary suppliers of NADPH for NO_3^- reduction in *A. nidulans*; 1) the high specific activities of G6PD and 6PGD compared to other $NADP^+$-linked dehydrogenases (Singh *et al.*, 1988), 2) the increased activity of G6PD, 6PGD and all other enzymes of the pentose phosphate pathway in mycelium grown on nitrate compared to urea (Carter and Bull, 1969: Hankinson and Cove, 1974) and 3) the observation that the pentose phosphate pathway mutants *ppp*A and *ppp*B are unable to utilise NO_3^- as sole nitrogen source (Hankinson, 1974).

The *ppp*A and *ppp*B mutants of *A. nidulans* are most probably defective in respectively transaldolase and a so far unidentified regulatory factor of the pentose phosphate pathway. Both mutations restrict the flow of metabolites through the pentose phosphate pathway and prevent growth on NO_3^- as sole nitrogen source (Hankinson, 1974).

Furthermore, the existence of a mannitol shuttle, which serves as an alternative source for NADPH

in the fungus *Alternaria alternata* (Hult and Gatenbeck, 1978) could not be demonstrated in *A. nidulans* (Singh *et al.*, 1985).
This again indicates that in *A. nidulans* the two oxidative reactions of the pentose phosphate pathway are the primary sources for NADPH.

The nitrate utilisation pathway in *Aspergillus nidulans* have been extensively investigated for more than three decades. Several mutants of the structural genes *nia*D and *nii*A encoding respectively nitrate and nitrite reductase, the genes involved in the synthesis of the nitrate reductase cofactor *cnx*, the nitrate permease gene *crn*A as well as the regulatory genes *nir*A and *are*A have been isolated, mapped and characterised (for reviews see Cove, 1979; Unkles, 1989). From the phenotypes of these mutants the following model for the regulation of the nitrate utilisation pathway has been deduced: The regulatory *nir*A gene product (NIRA) is constitutively expressed. An active form of NIRA induces transcription of the structural genes *nia*D and *nii*A. In the absence of NO_3^-, nitrate reductase inactivates NIRA so that it is no longer able to activate transcription. If however NO_3^- is present, NIRA is not inactivated by nitrate reductase leading to continued transcription and induced levels of the *nia*D and *nii*A gene products (Cove, 1979).

Apart from NO_3^- induction, the nitrate utilisation pathway is also subject to nitrogen metabolite repression, mediated by the *are*A gene product. The phenotypes of *are*A mutants suggest that its gene product (AREA) is essential for the transcription of a large number of genes, involved in the use of nitrogen sources other than ammonia or glutamine. Glutamine inactivates AREA, preventing the transcription of for example the *nia*D and *nii*A genes, even in the presence of NO_3^- (for a review see Tomsett, 1989; Caddick, 1992)

Recently the *nia*D, *nii*A (Johnstone *et al.*, 1990), *crn*A (Unkles *et al.*, 1991) genes and the regulatory genes *nir*A (Burger *et al.*, 1991a/b) and *are*A (Caddick *et al.*, 1986) were cloned and their nucleotide sequences were analysed. The cloning of these genes, especially the latter two, provided an opportunity to test the validity of the current model for the regulation of the nitrate utilisation pathway at the molecular level. Results reported so far, support the model proposed by Cove (Caddick, 1989; Burger *et al.*, 1991a/b); Hawker *et al.*, 1992). In addition these studies showed that not only *nia*D and *nii*A, but also *crn*A is regulated by *nir*A and *are*A (Unkles *et al.*, 1991; Hawker *et al.*, 1992).

By contrast, the genes involved in the generation of the NADPH, which in turn is required by nitrate reductase and nitrite reductase for the conversion of NO_3^- into ammonia, have not received much attention. These genes have not been cloned or studied at the molecular level and consequently little is known about their regulation.

The observation that the *nir*A gene product also mediates the twofold stimulation of the G6PD activity by NO_3^- (Hankinson and Cove 1974), indicates that the nitrate utilisation pathway and the pentose phosphate pathway are coregulated. The deduced amino acid sequence of the *nir*A gene shows several features of a DNA binding protein (Burger *et al.*, 1991a/b), which suggests that the increase in G6PD activity is regulated at the transcription level.

G6PD expression is a logical control site for *nir*A, because G6PD catalyses the first and committing step in the pentose phosphate pathway (Beutler and Kuhl, 1986). In order to investigate the regulation of NADPH production in *A. nidulans* we isolated and characterised the G6PD enzyme. Kinetic and inhibition studies of the enzyme suggested that the production of NADPH is its main physiological function (Wennekes *et al.*, 1993). Here we report on the cloning and analysis of the *A. nidulans gsd*A gene, encoding glucose-6-phosphate dehydrogenase.

Materials and methods

All chemicals used were analytical grade whenever commercially available. Radiochemicals were purchased from Amersham.

Plasmids, strains and growth conditions

The plasmids pGW635 (Goosen *et al.*, 1987), pGW101 (Wernars *et al.*, 1985), pGW175 (van den Broek *et al.*, 1995), pGW259 (van den Broek *et al.*, 1995), pHY201 (Yelton *et al.*, 1985) and pTZ-18u/19u (USB) were used in this study.
For transformation and propagation of plasmids *E. coli* DH5α (λ⁻, F⁻, *end*A1, *hsd*R17 (r_k^- m_k^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, ϕ80Δ*lac*ZM15) was used (Sambrook *et al.*, 1989). *E. coli* LE 392 (F⁻, λ⁻, *hsd*R514 (r_k^- m_k^+), *sup*E44, *sup*F58, *lac*Y1, *gal*K2, *gal*T22, *met*B1, *trp*R55) was used as host for recombinant λ phages (Murray *et al.*, 1977; Sambrook *et al.*, 1989).
A. nidulans WG096 *paba*A1, *y*A1 was used for library construction (Bos *et al.*, 1987) and *A. nidulans* WG328 *bi*A1, *arg*B2, *met*H2 for cotransformation experiments (Strain collection Agricultural University Wageningen). *A. niger* N593 *csp*A1, *pyr*A6 (Goosen *et al.*, 1987) was used for cotransformation experiments. Transformation of *A. nidulans* was performed as described for *A. niger* by van den Broek *et al.* (1995), except that the SMC buffer and the STC buffers contained 1.0 M Sorbitol instead of 1.3 M Sorbitol. *A. nidulans* and *A. niger* were grown as described by Pontecorvo *et al.* (1953).

Isolation and manipulation of nucleic acids

General manipulations of nucleic acids were performed according to Sambrook *et al.* (1989). Dideoxysequencing (Sanger *et al.*, 1977) on ds templates was performed with Taq DNA polymerase sequencing kits (Promega), according to the protocols from the supplier. Gel purified restriction fragments were labelled by the random primed labelling method (Feinberg and Vogelstein, 1983) as described by Sambrook *et al.* (1989). Chromosomal DNA was extracted from frozen mycelium as previously described (van den Broek *et al.*, 1995). For the construction of a genomic library the chromosomal DNA was purified further by CsCl gradient centrifugation. For Southern analysis, DNA was purified on Qiagen tube 20 as prescribed by the manufacturer (Diagen). λ DNA was purified with Qiagen tip 20 according to the manufacturer's protocol. RNA for primer extension and S1 assays was isolated as described by van Kan (1991).

Libraries and screening

The *A. nidulans* WG096 genomic library was constructed by cloning partial *Sau*3A digested DNA, size fractionated on sucrose gradients, in λEMBL3A arms (Frischauf *et al.*, 1983). The ligation mix was packaged using Gigapack Gold II extracts (Stratagene). After amplification the lysate was stored at 4°C. Plaque lifts, Southern blots and heterologous hybridisations were performed as previously described (van den Broek *et al.*, 1995).

Western blot and G6PD detection

Protein extractions from mycelium, enzyme assays, polyacrylamide electrophoresis, Western blotting and G6PD specific detection on blots were performed as reported earlier (Wennekes *et al.*, 1993).

Primer extension and S1 mapping

Primer extension on total RNA with the endlabelled oligonucleotide HLWLOO-1 (5'GCGAATTCT-GATCAGCATCTTATCGGGCGGAA 3') and TET-z reverse transcriptase was performed as described by van den Broek *et al.* (1995).
S1 was mapping was performed essentially as previously described (van den Broek *et al.*, 1995). The probe for S1 mapping was prepared with the HLWLOO-1 primer and the plasmid pGW514 (fig. 1). Prior to purification on a sequence gel, the probe was cut with 20 U *Dra*I for 30 min at 37°C.

Computer analysis

Computer assisted analysis of the DNA sequences was done using the GCG software package (Devereux *et al.*, 1984) on a VAX computer. The phylogenetic tree was constructed using the algorithm of Feng and Doolitlle (1987)

Results

Cloning of the gene.
To isolate the G6PD encoding gene from *A. nidulans*, a genomic library was screened with the 1.0 kb *Eco*RI-*Pst*I fragment encompassing the putative catalytic site of the *Drosophila melanogaster* G6PD enzyme (Jeffery *et al.*, 1985; Fouts *et al.*, 1988) at 50°C. Although the *D. melanogaster* probe had been successfully used to clone the *A. niger gsd*A gene (van den Broek *et al.*, 1995), no positive λ clones were isolated from the *A. nidulans* genomic library. However, probing at 55°C with the 1.8 kb *Nco*I fragment of the *A. niger gsd*A gene, which comprises almost the complete coding region, we could isolate several λ clones of its putative *A. nidulans* counterpart. Using smaller fragments from different parts of the *A. niger gsd*A gene as probes we mapped the putative *A. nidulans* G6PD encoding gene on the 4.8 kb *Sal*I-*Xho*I fragment of λ8.2 (fig. 1). The 5' proximal part of the gene was located in the vicinity of the *Sal*I site. The 4.8 kb *Sal*I-*Xho*I fragment was subcloned into pTZ19 to give pGW514 (fig.1).

Functionality of the putative *A. nidulans gsd*A gene

The plasmid pGW514 was introduced into *A. nidulans* WG328 by cotransformation with the *arg*B bearing plasmid pGW101, to establish the functionality of the cloned gene. Similarly, pGW514 was introduced into *A. niger* N593 with the *pyr*A containing plasmid pGW635.

Several *A. nidulans* and *A. niger* transformants exhibited an increased G6PD activity in protein extracts. Two *A. nidulans* and *A. niger* transformants with increased G6PD expression were subjected to Southern analysis to examine the presence of pGW514.

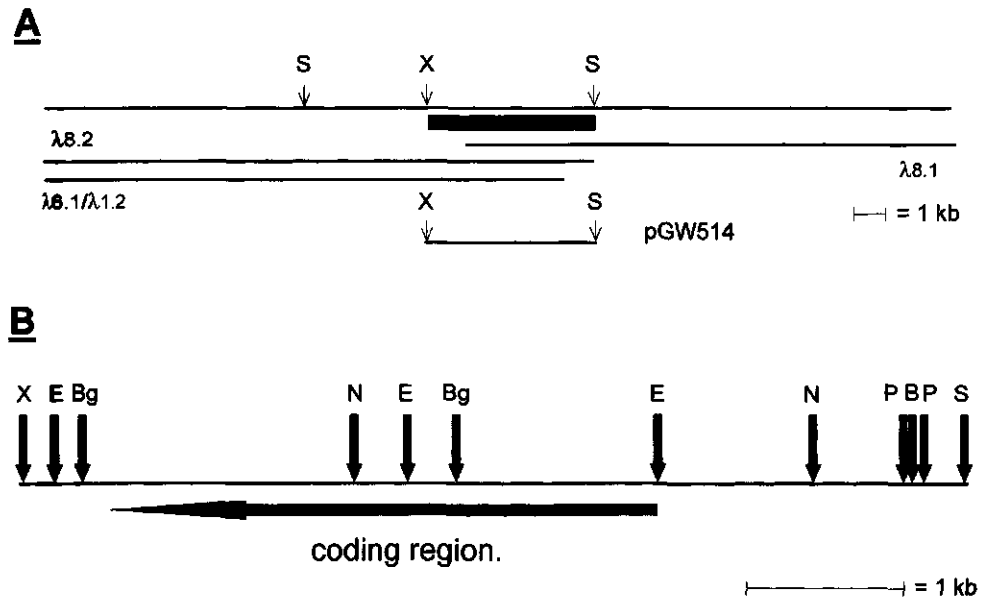


Figure 1. Restriction map of the *A. nidulans gsd*A gene.

Panel A: The λ clones containing the *A. nidulans gsd*A gene are indicated with thin lines. The regions that cross hybridised with the 1.8 bk *Nco*I fragment of the *A. niger gsd*A gene are marked by a black bar. Restriction sites S=*Sal*I and X=*Xho*I. **Panel B:** The 4.8 kb *Sal*I-*Xho*I fragment, indicated in panel A, was cloned into pTZ19 to give pGW514, which was used in the cotransformation experiments. Restriction sites B=*Bam*HI, Bg=*Bgl*II, E=*Eco*RI, N=*Nco*I, S=*Sal*I and X=*Xho*I.

The *A. nidulans* transformants V3.2 and IV2.1 showed a twofold increase in G6PD activity, compared to the wild-type control (table 1). The hybridisation pattern of these two transformants with the 1.8 kb *Bgl*II fragment of pGW514, were identical to the wild-type *A. nidulans* pattern (fig. 2: panel A, lanes 9, 10, 11 and 12). Using the *A. nidulans trp*C probe as an internal standard, we asserted that there was only one copy of pGW514 in both *A. nidulans* transformants. Because one copy of pGW154 corresponds with the observed twofold increase in G6PD activity, we assume that pGW514 contains a functional G6PD encoding gene from *A. nidulans*, which was named *gsd*A. Under stringent hybridisation conditions only weak signals are obtained with the *A. niger gsd*A 1.8 kb *Nco*I fragment in *A. nidulans* wild-type and transformants (fig. 2: panel B, lanes 9 to 14).

The presence of cross hybridising sequences in the wild-type *A. niger* (fig. 2: panel A, lanes 7 and 8), complicates the interpretation of the hybridisation patterns of the chromosomal DNA from the *A. niger* transformants. The 1.8 kb *Nco*I probe detects two larger *Nco*I fragments in *A. niger* N402 (fig. 2: panel B, lane 7), that cannot be assigned to the *A. niger gsd*A gene (van den Broek *et al.*, 1995). Furthermore, these two additional bands cross hybridise to the *A. nidulans gsd*A *Bgl*II fragment (fig. 2: panel A, lane 7). In the *Bgl*II digest of the chromosomal DNA of the two *A. niger* transformants, these cross hybridising bands coincide with 1.8 kb *Bgl*II fragment from pGW514. Fortunately the 2.2 kb *Nco*I fragment of pGW514 has no corresponding band in the *A. niger* wild type, thus allowing the detection of the cotransformed DNA in the *A. niger* transformants 5.3.1 and 5.10.4. From the comparison of hybridisation signals of the *A. niger trp*C gene (not shown) and the 0.66 kb *Bgl*II fragment of the *A. niger*

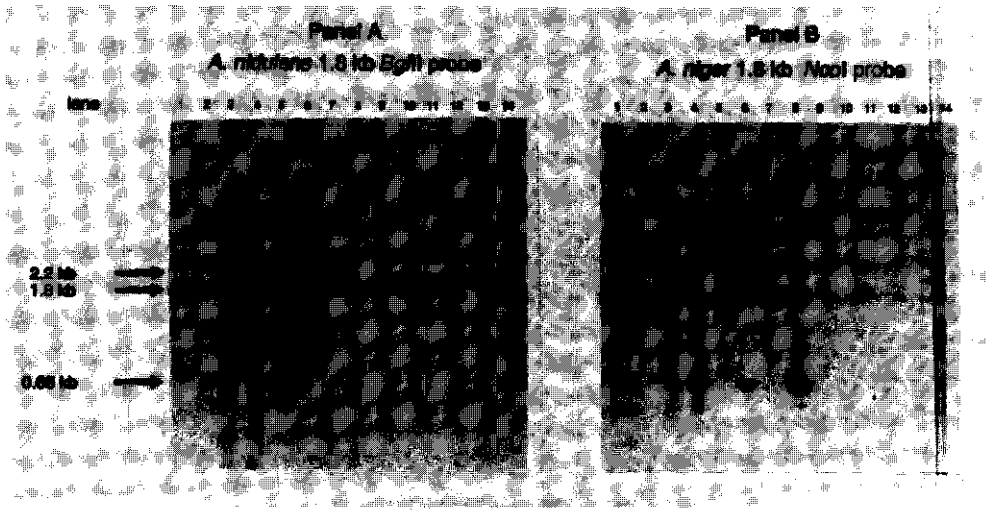


Figure 2. Southern analysis of G6PD overexpressing transformants.
Chromosomal DNA from wild-type *A. nidulans* (WG096) and two G6PD overproducing *A. nidulans* transformants (IV 2.1 and V 3.2, table 1) was digested with *Nco*I or *Bgl*II and hybridised with either the 1.8 kb *Bgl*II fragment of pGW514 (panel A lanes 9 to 14) or the 1.8 kb *Nco*I fragment (panel B lanes 9 to 14) of the *A. niger gsd*A gene (van den Broek *et al.*, 1995). Similarly chromosomal DNA of *A. niger* wild type (N402) and two G6PD overproducing transformants (5.3.1 and 5.10.4, table 1) was digested with *Nco*I or *Bgl*II and either hybridised with the 1.8 kb *Bgl*II fragment of pGW514 (panel A lanes 3 to 8) or the 1.8 kb *Nco*I fragment of the *A. niger gsd*A gene (panel B lanes 3 to 8). To estimate the number of integrated plasmids, the Southern blots were stripped and rehybridised with either the 0.8 kb *Hind*III fragment of the *A. niger trpC* (Kos *et al.*, 1985) gene or the *Eco*RI-*Bam*HI fragment of the *A. nidulans trpC* gene (Yelton *et al.*, 1984; Mullaney *et al.*, 1985). lane 1: 1.8 kb *Nco*I probe fragment, lane 2: empty, lane 3: *A. niger* transformant 5.10.4 *Nco*I digest, lane 4: *A. niger* transformant 5.10.4 *Bgl*II digest, lane 5: *A. niger* transformant 5.3.1 *Nco*I digest, lane 6: *A. niger* transformant 5.3.1 *Bgl*II digest, lane 7: *A. niger* N402 *Nco*I digest, lane 8: *A. niger* N402 *Bgl*II digest, lane 9: *A. nidulans* transformant V3.2 *Nco*I digest, lane 10: *A. nidulans* transformant V3.2 *Bgl*II digest, lane 11: *A. nidulans* transformant *Nco*I digest, lane 12: *A. nidulans* transformant IV2.1 *Bgl*II digest, lane 13: *A. nidulans* 096 *Nco*I digest, lane 14: *A. nidulans* 096 *Bgl*II digest.

Tabel 1. G6PD activity in *A. nidulans* and *A. niger* transformants

Strain	G6PD activity μmoles NADPH/min/mg protein	A. nidulans *gsd*A copy number
A. nidulans WG096	1.4	1
transformant IV 2.1	2.8	2
transformant V 3.2	2.1	2
A. niger N402	1.0	0
transformant 5.3.1	1.9	ND
transformant 5.10.4	1.4	ND

*gsd*A gene (fig. 2: panel B, lanes 4, 6 and 8) with the signal of the 2.2 kb *Nco*I fragment from the *A. nidulans gsd*A gene (fig. 2: panel A, lanes 3 and 5), we concluded that *A. niger* transformant 5.3.1 contains more copies of pGW514 than transformant 5.10.4. Transformant 5.3.1 also shows a higher G6PD activity in protein extracts, than transformant 5.10.4 (table 1). Since we had no single copy reference the absolute copy number of pGW514 in *A. niger* transformants could not be determined. In any case the observations indicate, that the *A. nidulans gsd*A gene is expressed when introduced into *A. niger*.

Screening for *gsd*A related sequences in *A. nidulans* and *A. niger*

Southern blots of *Bgl*II and *Sal*I digests of *A. nidulans* chromosomal DNA and *Nco*I and *Bam*HI digests of *A. niger* chromosomal DNA were prepared. Identical blots were hybridised both at 68°C and 58°C with either the 1.8 kb *Bgl*II fragment of pGW514 or the 1.8 kb *Nco*I fragment of the *A. niger gsd*A gene (fig. 3). With both probes only the predicted 1.8 kb *Bgl*II and 9 kb *Sal*I fragments in *A. nidulans* hybridised. This strongly indicates that in *A. nidulans* G6PD is encoded by a single gene. As reported previously (van den Broek *et al.*, 1995) in *A. niger* not only the expected 1.8 kb *Nco*I fragment, but also two bands of higher molecular weight hybridise to the *A. niger gsd*A *Nco*I fragment.

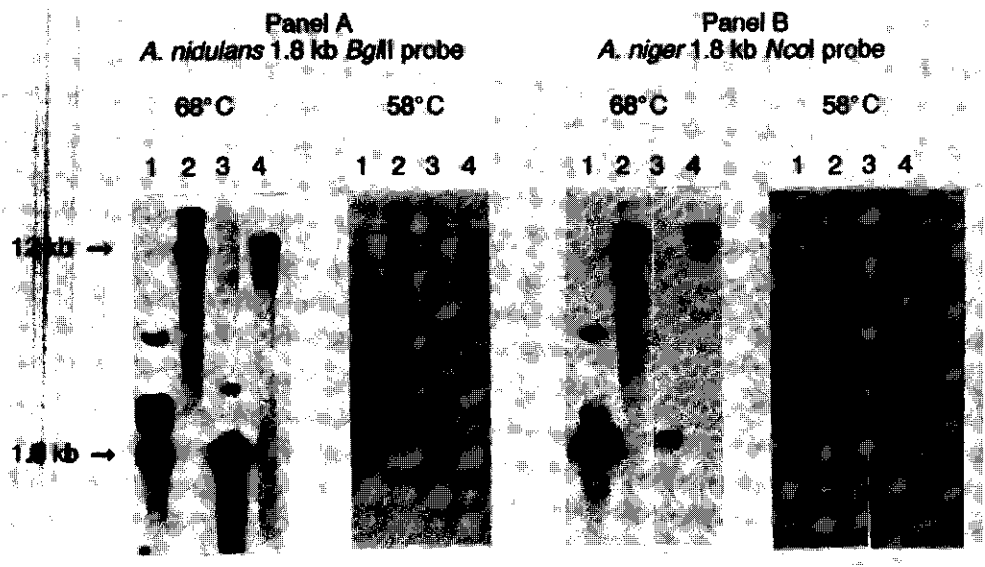
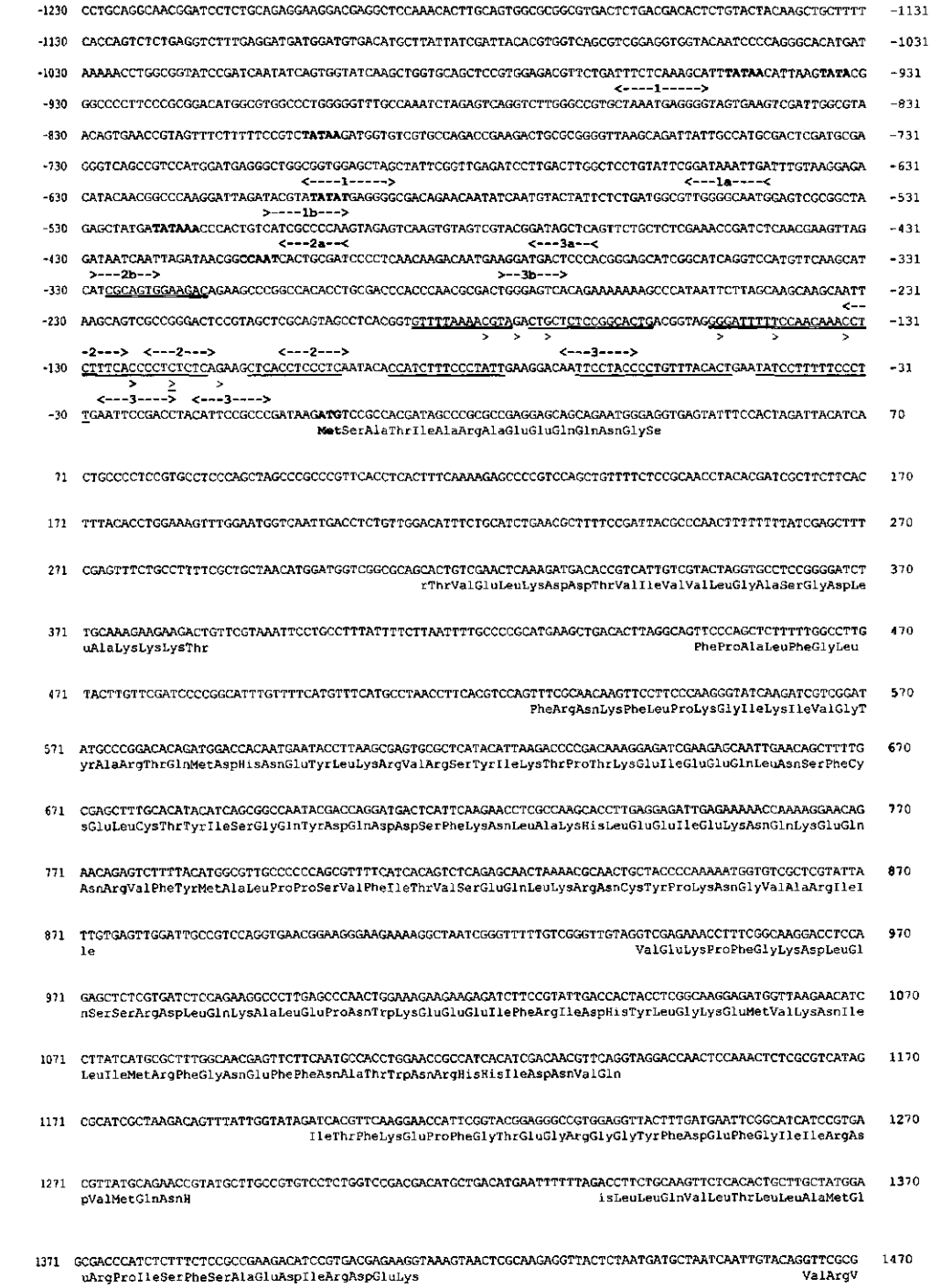


Figure 3. Screening for sequences related to the *A. nidulans gsd*A gene.
Identical Southern blots were prepared from chromosomal DNA of the *A. nidulans* strain 096, digested with *Sal*I or *Bgl*II, and from the *A. niger* strain N402, digested with *Bam*HI or *Nco*I. The blots were hybridised at 58°C and 68°C either with ^{32}P-labelled 1.8 kb *Bgl*II *A. nidulans gsd*A fragment or 1.8 kb *Nco*I *A. niger gsd*A fragment. **Panel A:** *A. nidulans* probe lane 1: *Bam*HI digest *A. niger* DNA, lane 2: *Nco*I digest *A.niger* DNA, lane 3: *A. nidulans* DNA *Bgl*II digest, lane 4: *A. nidulans* DNA *Sal*I digest. **Panel B**: *A. niger probe* lane 1: *Bam*HI digest *A.niger* DNA, Lane 2: *Nco*I digest *A.niger* DNA, lane 3: *A. nidulans* DNA *Bgl*II digest, lane 4: *A. nidulans* DNA *Sal*I digest. The *A. niger Nco*I fragment and the *A. nidulans Bgl*II fragment are not completely equivalent: the position of the *Nco*I fragment is more upstream in the coding region than the *Bgl*II fragment. Therefore if the *A. niger* probe is used to probe *A. nidulans* genomic DNA or vice versa, additional bands will hybridise.



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1471  TCTTGCGCGCCATGGACCCCATTCAACCCAAGGATGTCATCATCGGCCAGTACGGCAGATCACTAGATGGTAGCAAACCCGCATATAAGGAGGATGATAC  1570
      alLeuArgAlaMetAspProIleGlnProLysAspValIleIleGlyGlnTyrGlyArgSerLeuAspGlySerLysProAlaTyrLysGluAspAspTh

1571  AGTGCCACAGGACTCTCGCTGCCCCACCTTCTGTGCGCTGGTCGCTCACATCAAGAACGAGAGATGGGATGGTGTTCCTTTCATCATGAAGGCGGGTAAA  1670
      rValProGlnAspSerArgCysProThrPheCysAlaLeuValAlaHisIleLysAsnGluArgTrpAspGlyValProPheIleMetLysAlaGlyLys

1671  GGTATGCAGACCTGATACATGCCTGATGTACCATGCACTCACCATCTCTAGCCCTCAACGAACAGAAGACCGAAATCCGTATTCAGTTCAAGGACGTCAC  1770
      A                                                    laLeuAsnGluGlnLysThrGluIleArgIleGlnPheLysAspValTh

1771  TTCTGGCATCTTCAAGGATATCCCCCGCAACGAGCTTGTCATCCGTGTCCAACCCAACGAATCTGTCTACATCAAGATGAACTCGAAACTTCCTGGACTT  1870
      rSerGlyIlePheLysAspIleProArgAsnGluLeuValIleArgValGlnProAsnGluSerValTyrIleLysMetAsnSerLysLeuProGlyLeu

1871  TCCATGCAGACCGTGGTTACTGAGCTGGATCTCACCTACCGTCGTCGATTCTCCGACCTCAAGATTCCTGAAGCCTACGAATCTTTGATCTTGGATGCTC  1970
      SerMetGlnThrValValThrGluLeuAspLeuThrTyrArgArgArgPheSerAspLeuLysIleProGluAlaTyrGluSerLeuIleLeuAspAlaL

1971  TCAAAGGAGACCACTCCAACTTTGTCCGTGACGACGAGCTCGATGCTAGCTGGAGGATGTTCACTCCTCTGCTCCACTACCTCGATGACAACAAGGAGAT  2070
      euLysGlyAspHisSerAsnPheValArgAspAspGluLeuAspAlaSerTrpArgMetPheThrProLeuLeuHisTyrLeuAspAspAsnLysGluIl

2071  CATCCCTATGGAATACCCCTACGGTAAGCCGAAGCTTGTAGATTGACCTCTTGGGTTTCGCCGCTAACGAATGCTCCTAGGTTCCCGCGGGCCCTCCGTT  2170
      eIleProMetGluTyrProTyrG                                                    lySerArgGlyProSerVal

2171  CTTGACGACTTCACCGCTTCCTATGGCTACAAGTTCAGTGATGCTGCGGGATACCAGTGGCCGTTGACGCACACTACGCCTAACAGGCTGTAGAGGTTGT  2270
      LeuAspAspPheThrAlaSerTyrGlyTyrLysPheSerAspAlaAlaGlyTyrGlnTrpProLeuThrHisThrThrProAsnArgLeuEnd

2271  AGTTGTATAGGGGAACTGTTTGCAAGGACAAGAGCGTCTGGGTTTACGGCGTCTATCGTTTTGAAAGGAAAATGAGAGCTTAATGTTAACACGACTGGTA  2370

2371  TCTATCTAAAGATGACATGGAGTCATGTGAAGCTACATGATTGGGCTGAGCGGCTGTTGATTATAGTTATGCTATATACAACCAAGCATTTGAAATCCGA  2470

2471  CACGTTTCATGTTTGCAAGACCCCTGACCATTAAGTGACACGATACTGCAAGTAGGATAAACTTCGCACTCGTAATGAGCTCAGGCCTATGCTTTTAGTG  2570
2571  GGTGGGTCACTTAACGCCGGACGGGAAAATTACTGTACGGTCGGGAGTTGACTAGCCCGCATGCAGCCTGCCTGGCCTGCGCTGCACCATCAACAACAGC  2670
2671  CCGCCCGTTTTCTGACCGTATACACCTTTGTCCTTCATTATTGTTCCCCCTTCGCGTTACCCCCCGGTCTATACACTCTTTCAGCGGCTATACCAAAAGA  2770
2771  TCTCAACTATCTGCCCTAGAATTCCGCGCCCCTGGACTCGCAATGTCTTCGAAACGCCCGTGCGCACCTTGATCTCATTTATCCACATTCTTGATCGCTA  2870
2871  TTTTTATCCCCTTTATAGCTCCCTAATCGTGAGACAACCGTCCAGGTCCCCATATCCCCGGAAATTCGTCAAGCATCGCCGG                    2952
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Figure 4. Nucleotide and deduced amino acid sequence of the *A. nidulans gsd*A gene.

The positions of the putative introns were determined on the basis of homology to the intron-exon boundary consensus sequence (Rambosek and Leach, 1987) and by alignment to the *A. niger* sequence. The transcription starts were determined by a S1 nuclease protection assay are indicated by > signs and the one mapped by primer extension is marked by a ≥. The translation start, stop codon and sequences homologous to TATA and CAAT boxes are indicated in bold print. C+T rich sequences in the direct vicinity of the transcription starts are underlined. The inverted repeats (1a-1b, 2a-2b, 3a-3b) and the direct repeats (1, 2 and 3) are indicated with dashed arrows. In the 3' non-translated trailer, the sequence fitting the consensus $CA^{T}/_{C}A^{T}/_{G}G$, thought to play a role in polyadenylation is doubly underlined. The sequences conserved in the 3' non-translated trailer of the *A. niger gsd*A gene are underlined.

Nucleotide sequence

The nucleotide sequence of the *A. nidulans gsd*A gene was determined according to a sequencing strategy in which both strands of partially overlapping subclones were analyzed. The results, together with the deduced amino acid sequences, putative intron positions and transcription start points (tsp) are summarised in figure 4.

Introns

Comparison of the *A. nidulans gsd*A sequence with the *A. niger* genomic sequence (van den Broek *et al.*, 1995) and the intron-exon border consensus (Rambosek and Leach, 1987) 5'-$GT^{G}/_{A}$NGT..

Figure 6. Intron-exon boundary sequences in the Aspergillus *gsda* genes.

Species	Intron	5'	Lariat	3'	Intron length	Intron homology
A. niger	1	GTGAGT	AGCTAACA	CAG	348	60.8%
A. nidulans		GTGAGT	TGCTAACA	CAG	269	
A. niger	2	GT**C**AGT	AGCTAACC	CAG	55	63.0%
A. nidulans		GT**T**CGT	AGCTGACA	CAG	62	
A. niger	3	GTATGT	TGCTGA**T**T	CAG	65	56.9%
A. nidulans		GTAC**T**	C**C**CTAACC	CAG	58	
A. niger	4	GTGAGT	T**T**CT**T**ACT	CAG	71	47.8%
A. nidulans		GTGAGT	GGCTAA**T**C	TAG	69	
A. niger	5	GTACGA	TACT**TG**CT	TAG	65	42,4%
A, nidulans		GTAGGA	CGCTAA**G**A	TAG	60	
A, niger	6	GTACGT	TGCTGA**T**A	TAG	58	61.5%
A. nidulans		GTATGC	TGCTGACA	TAG	52	
A. niger	7	GTAC**A**GT	TACTGACA	CAG	52	56.3%
A,. nidulans		GTAA**A**GT	TGCTAA**T**C	CAG	56	
A. niger	8	GTATGT	TACTAATA	CAG	50	50.0%
A. nidulans		GTATG**C**	CACT**C**ACC	TAG	50	
A. niger	9	GTACGT	TACTGACC	CAG	55	51.9%
A. nidulans		GTAAG**C**	CGCTAACG	TAG	57	
Consensus		GT$^{G}/_{A}$NGT	$^{G}/_{A}$CT$^{C}/_{A}$AC	$^{C}/_{T}$AG		

The nucleotides deviating from the intron-exon boundary consensus (Rambosek and Leach, 1987; Gurr 1987; Gurr 1987) are underlined in bold print. The lengths of the introns and the sequence homology of the corresponding introns from *A. nidulans* and *A. niger* are also indicated.

$^{G}/_{A}$CT$^{C}/_{A}$ACN..$^{C}/_{T}$AG-3', enabled us to determine the positions of the putative introns in the *A. nidulans* G6PD sequence without a matching cDNA sequence. The alignment with the *A. niger gsd*A gene also revealed that the positions of the putative introns had been exactly conserved with respect to the coding regions (fig. 5). Intron 7 of both *Aspergillus gsd*A genes has the same position in the coding region as intron number 8 does in the human G6PD gene (Chen *et al.*, 1991). None of the other introns shows such a position conservation compared to introns in the human and the Drosophila G6PD gene (Fouts *et al.*, 1988; Chen *et al.*, 1991).

No obvious splice signals occur in either gene between the ATG and the transcription start and therefore the *A. nidulans gsd*A gene probably contains nine introns, just like the *A. niger gsd*A gene.

Like in the *A. niger gsd*A gene, most aberrant intron-exon boundary sequences are the result of single base substitutions in the consensus sequence. The only exception is the supposed donor splice site of intron number 7, that carries an additional A. It is remarkable that the aberrant donor splice sites of introns 5 and 7 have been conserved in the *gsd*A genes of both Aspergillus species (fig. 6). Like in the *A. niger gsd*A gene the first intron in *A. nidulans* is exceptionally long: 269 bp. Gurr *et al.*, 1987; Rambosek and Leach, 1987). The other introns are considerably smaller: 50 to 69 bp long. This length is in the same range as the intron size in most genes from filamentous fungi (Unkles, 1992).

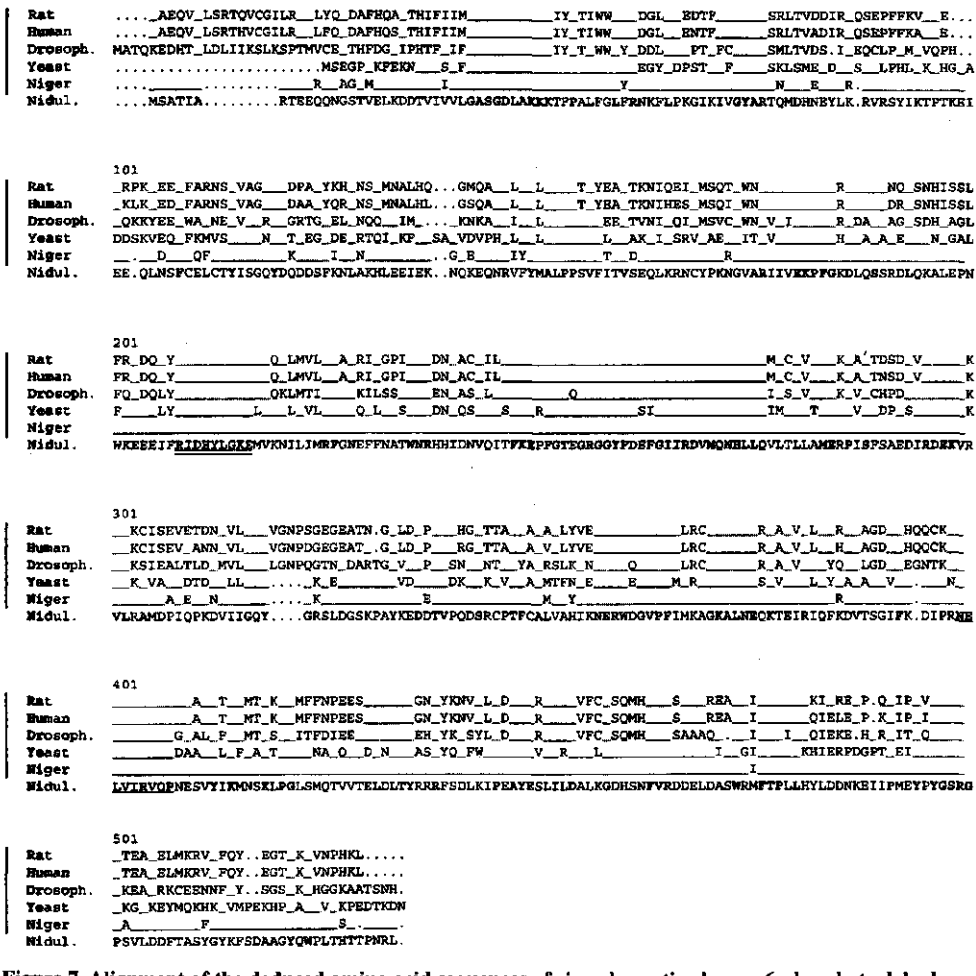

Figure 7. Alignment of the deduced amino acid sequences of six eukaryotic glucose-6-phosphate dehydrogenase encoding genes. The deduced amino acid sequences of human, rat, *D. melanogaster*, *S. cerevisiae*, *A. nidulans* and *A. niger* G6PD encoding genes were aligned according to the algorithm of Needleman and Wunsch (1970). Gaps introduced for optimal alignment are indicated with dots. Amino acid residues identical to those in the *A. nidulans* sequence are indicated by horizontal bars and those conserved in all sequences are indicated in bold print. The putative catalytic site is doubly underlined and the putative NADPH binding site is underlined.

Deduced amino acid sequence

Because an *A. nidulans gsd*A cDNA clone was not available, we assigned the initiation codon on the basis of comparison with the *A. niger gsd*A gene. No other ATG is found in the 5' proximal region, or any in frame ATG downstream of the putative initiation codon. The *A. nidulans gsd*A open reading frame was found to encode a 58.626 kD protein, which agrees with the molecular weight of 60 and 57 kD estimated from SDS-PAA gels for the two *A. nidulans* G6PD monomers (Wennekes *et al.*, 1993).

The deduced amino acid sequences of *A. niger* and *A. nidulans gsd*A are 92.2% identical and, taking

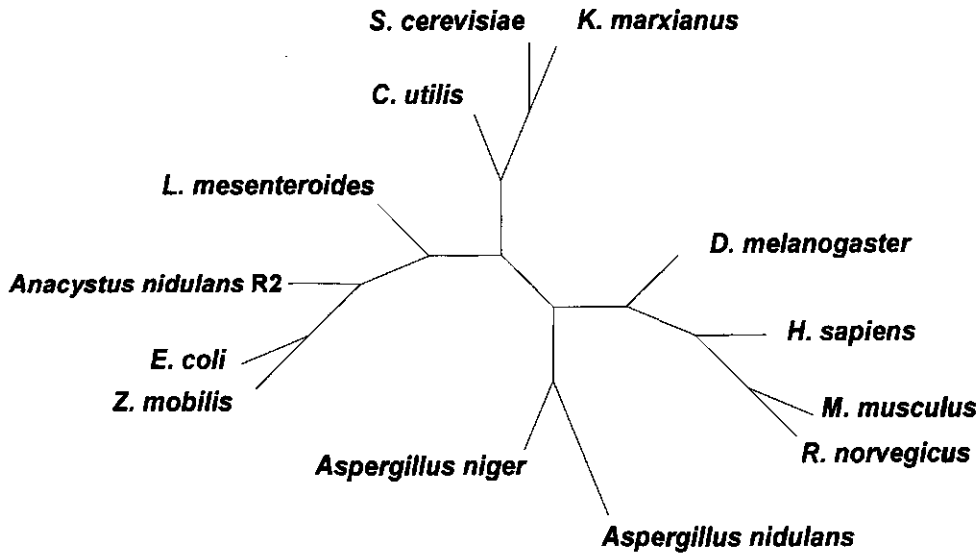

Figure 8. Phylogenetic tree based on the homology of the deduced amino acid sequences of G6PD encoding genes. Alignments of the deduced amino acid sequences of the G6PD encoding genes of *Leuconostoc mesenteroïdes* (Lee *et al.*, 1991), *Zymomonas mobilis* (Barnell *et al.*, 1990), *Escherichia coli* (Rowley *et al.*, 1990), *Anacystis nidulans* (Scanlan *et al.*, 1992), *Saccharomyces cerevisiae* (Nogae and Johnstone, 1990), *Candida utilis* (Jeffery *et al.*, 1993), *Klyveromyces marxianus* (Wesolowski-Louvel *et al.*, Pers. Comm.), *Rattus norvegicus* (Ho *et al.*, 1988), *Drosophila melanogaster* (Fouts *et al.*, 1988), *Homo sapiens* (Persico *et al.*, 1986), *Aspergillus niger* (van den Broek *et al.*, 1995) and *Aspergillus nidulans*, were used to calculate an unrooted tree using the bootstrap (N=10) parsimony method of Fehlenstein (1989), implicated in the PHYLIP programme.

into account conservative substitutions, even have a similarity of 96.5%. This high degree of conservation explains the strong cross reaction of an *A. nidulans* G6PD antiserum with *A. niger* G6PD on Western blots (Wennekes *et al.*, 1993).

Comparison of the deduced amino acid sequences shows that the Aspergillus G6PDs are not only very homologous to each other, but also to other eukaryotic G6PDs (fig. 7). Alignment of the deduced amino acid sequences from the *A. niger* and *A. nidulans* genes with other G6PDs also allows functionally important domains to be localised. The putative catalytic domain with the reactive Lys 215, characterised in the yeast and human enzyme (Jeffery *et al.*, 1985; Camardella *et al.*, 1988; Bergman *et al.*, 1991) as well as the putative $NADP^+$ binding domain, mapped in human G6PD (Hirono *et al.*, 1989), are also preserved in the two Aspergillus enzymes.

The homology among G6PDs extends even to those of prokaryotes. This makes G6PD protein sequences a useful tool to study phylogenetic relations both between closely related and between more distant organisms. As an example a phylogenetic tree was constructed with several eukaryotic and prokaryotic protein sequences which is depicted in figure 8.

3' end non-translated sequences

Little is known about the sequences involved in polyadenylation and transcription termination in filamentous fungi (Unkles, 1992). In mammalian genes sequences with the consensus $AAT^{A}/_{T}AA$ (Proudfoot and Brownlee, 1976; Benoist *et al.*, 1980) and the consensus $CA^{T}/_{C}{}^{A}/_{G}G$ (Berget, 1984) serve as polyadenylation signals. Originally these sequences were identified by comparing the 3' proximal regions of a number of mammalian genes.

Like many genes from filamentous fungi, lacks the *A. nidulans gsd*A gene AATAAA-like polyadenylation signals (Gurr, 1987; Unkles, 1992). But the sequence CATGG (fig. 4: pos. 2385), fitting the consensus $CA^{T}/_{C}{}^{A}/_{G}G$ (Berget, 1984), is present in the 3' non-translated trailer of *A. nidulans gsd*A as well as in the *A. niger gsd*A gene.

Downstream of the open reading frame the *A. nidulans* and *A. niger gsd*A sequences diverge sharply. The 3' proximal regions have only two short sequence motifs in common; one perfectly conserved sequence ATGTTAA and one with the sequence $ATA^{A}/_{G}GGG$ (fig. 4: pos. 2277). In the *A. niger gsd*A gene the sequence ATAAGGG coincides with the stop codon, and in *A. nidulans* the motif ATAGGGG is located 16 bp downstream of the stopcodon (pos. 2261). The ATGTTAA motif resides in *A. nidulans* 26 bp and in *A. niger* 24 bp downstream of the $CA^{T}/_{C}{}^{A}/_{G}G$ consensus. Because these conserved sequences occupy similar positions in both *gsd*A genes, they could play a role in polyadenylation or transcription termination.

Transcription start and 5' non-transcribed sequences

Computer analysis of the sequence 1000 bp upstream of the initiation codon shows the presence of several direct and inverted repeats (fig. 4) in *A. nidulans*. None of these repeated sequences are also present in the promoter of the *A. niger gsd*A gene. Alignment of the 5' proximal regions of the *A. nidulans* and *A. niger gsd*A genes reveals four highly conserved motifs with a length of respectively 12, 13, 15 and 20 bp (fig. 4 pos. -345 to -333, -187 to -174, -172 to -157 and -151 to -131). In both *gsd*A genes these sequences are present in the same order. With the S1 nuclease protection assay eight transcription start sites, differing in intensity, were detected between position -179 and -113 (fig. 4 and 9). The major transcription start was mapped to position -124.

No clear TATA or CCAAT homologies are present within 110 bp upstream of the major transcription (pos. -124) start site. There are several short C+T rich sequences, which contain most of the major transcription starts. The primer extension with TET-z on *A. nidulans* RNA reveals several stops (not shown), mapping in the first C+T rich sequence upstream of the ATG. The largest extension product mapped to position -119, which is near the major transcription start determined with S1.

Discussion

The *A. nidulans* glucose-6-phosphate dehydrogenase encoding gene was isolated by heterologous hybridisation with the *A. niger gsd*A gene. Comparison of the *Aspergillus gsd*A sequences showed that in the coding region homology between the *A. niger* and *A. nidulans gsd*A genes at the DNA level is 85%. In the introns, 5' and 3' flanking regions this homology decreases and varies from 60% to 40%.
Comparison of the *D. melanogaster* 1.0 kb *Eco*RI-*Pst*I fragment to the corresponding part of the *A. nidulans* and *A. niger gsd*A genes, revealed no obvious difference in the overall (36-40%) or local ho-

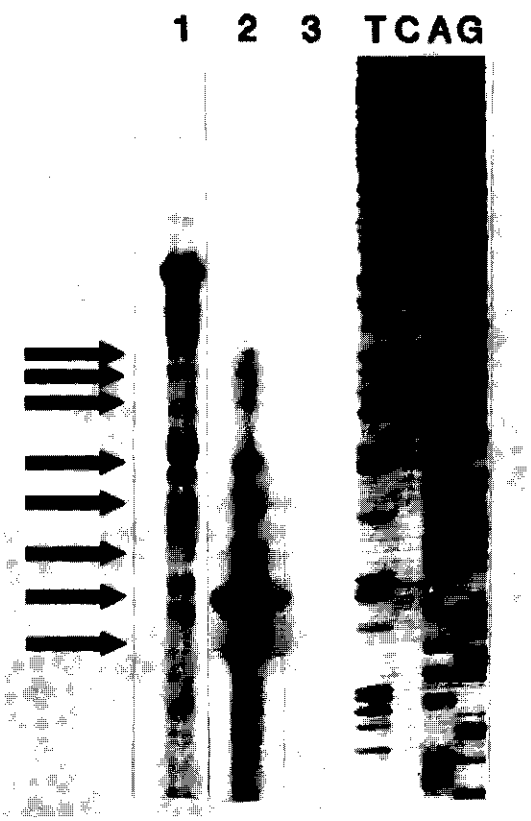

Figure 9. Mapping of the transcription start points in the *A. nidulans gsd*A gene.
S1 nuclease protection assay. Lane 1: probe, Lane 2: S1 probe hybridised to 50 µg total yeast RNA and treated with S1 nuclease. Lane 3: S1 probe hybridised to 50 µg total *A. nidulans* RNA and treated with S1 nuclease. Lane 4 to 7: sequence ladder. The probe was synthesised using the HLWLOO-1 oligonucleotide that contained 14 non matching base pairs to its 5' end. The sequence ladder was generated with the same primer and consequently 14 bp have to be added to the observed start site in order to obtain the true transcription start.

mology. This means that our inability to isolate the *A. nidulans gsd*A gene with the *D. melanogaster* probe must have been a mere coincidence.

Both the number of the introns and their positions in the *Aspergillus gsd*A genes are identical. The latter is often observed for corresponding genes in different species of filamentous fungi (Unkles, 1992). However, in the G6PD encoding gene (*ZWF1*) of the related ascomycete *S. cerevisiae* (Nogae and Johnston, 1990) no introns are found. This complete absence of introns makes the *S. cerevisiae ZWF1* gene the exception among the eukaryotic G6PD genes; for *D. melanogaster Zw* has three (Fouts *et al.*, 1988), *A. niger* and *A. nidulans gsd*A both nine and the human G6PD encoding gene (*Gd*) has twelve introns (Chen *et al.*, 1991). The absence of general position conservation of introns among G6PD genes, contradicts the view that exons comprise functional domains (Gilbert *et al.*, 1986), especially since the deduced amino acid sequences exhibit extensive homology.

Heterologous hybridisation with the *A. nidulans* and *A. niger gsd*A gene revealed no other G6PD encoding genes in *A. nidulans* chromosomal DNA and therefore the two G6PD monomers in pure enzyme preparations (Wennekes *et al.*, 1993), must be derived from the *gsd*A gene. Since there is no alternative initiation codon, the two *A. nidulans* G6PD monomers cannot be explained by the alternative use of two initiation codons as proposed for *A. niger gsd*A (van den Broek *et al.*, 1995). Consequently the origin of the two *A. nidulans* G6PD monomers must be either proteolytic degradation during isolation or post-translational modification. Both the *A. nidulans* and *A. niger gsd*A genes encode a protein

of about 59 kD, but the molecular weights estimated from SDS-polyacrylamide gels differ significantly; 60 and 57 kD for *A. nidulans* but only 55 and 53 kD for *A. niger*. This suggests that the *A. niger gsd*A gene product is more extensively modified or degraded than the *A. nidulans gsd*A gene product.

Several blocks of perfect conservation are present throughout the eukaryotic amino acid sequences. It is reasonable to assume that highly conserved domains play a role in for example substrate and/or cofactor binding and multimerisation. For dinucleotide cofactor (NAD^+, $NADP^+$ and FAD^+) binding sites two characteristics have been reported; GXXGXXG/A motifs located in a ß-α-ß secondary structure arrangement and charged amino acids C-terminal of the ß-α-ß fold (Ohlsson *et al.*, 1974; Rossmann *et al.*, 1974; Wieringa *et al.*, 1985). In all prokaryotic and eukaryotic sequences, including both Aspergilli, two of these sequence motifs GXXGXXG/A (fig. 7 pos 42-48 and 242-248) are present in a highly conserved sequence context. The Arg and His residues, that are C-terminal of the ß-α-ß fold are thought to interact with the 2'-phosphate group of the NADPH (Wieringa *et al.* 1984). The conserved Arg residues in positions 61, 76, 267 and the His residue in position 273 are C-terminal of one of the GXXGXXG/A motives and could therefore contribute to $NADP^+$ binding.
In human G6PD, amino acid substitutions in the region between positions 384 and 425 lead to reduced affinity for $NADP^+$ (Hirono *et al.*, 1989). The amino acid residues in this putative $NADP^+$ binding domain have a perfect match in both Aspergillus sequences and all other eukaryotic G6PDs. This domain does not contain a GXXGXXG/A motif, but computer analysis predicts a ß-α-ß secondary structure (Persson *et al.* 1991). This putative $NADP^+$ binding domain is not conserved in the deduced amino acid sequences from *Escherichia coli, Leuconostoc mesenteroïdes* and *Zymomonas mobilis* (Rowley *et al.*, 1991; Lee *et al.*,1991; Barnell *et al.*, 1990). A possible explanation for this is, that the enzymes encoded by the *zwf* genes of the latter two are able to use both NAD^+ and $NADP^+$ as cofactor (Lee *et al.*,1991; Barnell *et al.*, 1990). In general homology is lower among prokaryotic G6PD sequences, as compared to eukaryotic G6PDs.

Abolishment of enzyme activity by acetyl-acetic acid and pyridoxyl 5'-phosphate, as a result of specific modification of the Lys 215 residue in human and yeast G6PD, lead to the identification of this residue as part of the catalytic site (Jeffery *et al.*, 1985; Camardella *et al.*, 1988). The observation that G6P, but not $NADP^+$, protects G6PD from inactivation by pyridoxal 5'-phosphate, suggests that the lysyl residue is involved in G6P binding (Camardella *et al.*, 1988). Although both Aspergillus enzymes are not inhibited to any extent *in vitro* by acetyl-salicylic acid (Wennekes *et al.*, 1993), the amino acid sequences around Lys 215 are strictly conserved in both Aspergilli as they are in all G6PDs analyzed to date.
The fact that G6PD is an ubiquitous enzyme and the fact that its protein sequences are conserved over a wide range of genera, makes it a suitable target for phylogenetic analysis. With primers complementary to conserved regions, portions of the gene can be amplified and sequenced, analogous to the thus far more commonly used rRNA genes (White *et al.*, 1990).

In *A. nidulans* the region upstream of the ATG has multiple short C+T rich stretches, where as *A. niger* has three fairly long C+T rich sequences in which all three transcription starts map. In *A. nidulans* no relation between the position of the transcription start and the short C+T rich sequences is evident. Only the two major start sites map in or downstream of a C+T rich sequence. This suggest a relation between the length of CT boxes and control on transcription initiation.
The discrepancy between the primer extension and S1 protection assay is caused by the apparent inability of TET-z reverse transcriptase to efficiently transcribe C+T rich sequences. A similar observation was made in the primer extension of the *A. niger gsd*A gene (van den Broek *et al.*, 1995).

Introduction of the cloned *A. nidulans gsd*A gene into *A. nidulans* and *A. niger* results in increased G6PD activity. The latter indicates that the *A. nidulans gsd*A gene is expressed in *A. niger*, suggesting

the presence of conserved control elements in the promoters. Highly overproducing transformants obtained with the *A. niger gsd*A gene are severely inhibited in their growth (van den Broek *et al.*, 1995). The overexpression of G6PD obtained with *A. nidulans gsd*A transformants has no significant effect on fungal growth. However, none of the *A. nidulans* transformants was highly overexpressing or contained more than one extra *gsd*A copy, which might indicate that only high overexpression is lethal.
The direct and inverted repeats detected in the *A. nidulans gsd*A promoter cannot be found in the *A. niger gsd*A promoter nor are the repeats in the *A. niger gsd*A promoter present in its *A. nidulans* counterpart. Assuming a similar regulation of the *Aspergillus gsd*A genes, is it questionable whether these repeats act as binding sites for trans-acting factors. Consequently the four conserved sequence motifs in the *gsd*A genes could constitute or contain functional promoter elements. Highly conserved sequences in the 5' proximal regions of the *A. nidulans* and *A. niger gpd*A and *oli*C genes have been found to contain elements affecting transcription (Punt and van den Hondel, 1992).

The coregulation of *gsd*A gene and the nitrate utilisation genes by *nir*A also implies, that the for example the *nia*D gene and the *gsd*A gene should have common promoter elements. The four conserved sequence motifs in the *A. nidulans gsd*A promoter are larger than the motifs in the 5' proximal regions of the nitrate utilisation genes in *A. nidulans* and do not exhibit any resemblance to these motifs Unkles *et al.*, 1992). Since binding sites of trans acting factors can be as small as 5 to 6 bp and sometimes have degenerate sequences (Punt and van den Hondel, 1992), important promoter elements may not be identified by alignment of 5' proximal regions. Therefore a functional analysis of the *A. nidulans* and *A. niger gsd*A promoters is required to study the function of the conserved sequences and to identify the nitrate responsive elements.

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Chapter 5 Studies on the regulation of the expression of the *Aspergillus niger gsd*A gene, encoding glucose-6-phosphate dehydrogenase.

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Summary

This chapter describes the functional analysis of the promoter from the *A. niger gsd*A gene, which encodes glucose-6-phosphate dehydrogenase. For this purpose we used a reporter construct consisting of 990 bp of the *gsd*A 5' proximal region and its start codon fused to the *E. coli lac*Z gene. Deletion analysis showed that the sequences between -298 and the ATG (+1) are indispensable for *gsd*A transcription. This region contains four short sequence motifs, that have been conserved between the *A. niger* and *A. nidulans gsd*A promoters. Upstream of -298 we found positive acting promoter elements. Northern blot and G6PD enzyme activity analysis showed, that the presence of a *gsd*A promoter-*lac*Z fusion in transformants does not interfere with expression of the resident *gsd*A gene. The expression of the *A. niger gsd*A gene is not modulated at the transcriptional level, the steady state mRNA level or at the G6PD enzyme activity level, by compounds that have been reported to alter the NADPH/NADP$^+$ ratio, like NO_3^-, NH_4^+ or paraquat. Furthermore, *A. niger gsd*A expression is not affected by the presence of methionine in the growth medium as has been reported for the *S. cerevisiae* glucose-6-phosphate dehydrogenase encoding gene, *ZWF1*. The data from the promoter deletion study fit the observations of the expression of the resident *gsd*A gene by Northern blot and G6PD enzyme analysis. However, during growth on D-xylose G6PD activity and *gsd*A mRNA levels do not change compared to the level on glucose, but the transcription of the *gsd*A-*lac*Z fusion decreases. An explanation for this discrepancy is proposed.

Introduction

Recently the glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate:NADP$^+$ oxidoreductase, EC 1.1.1.49) encoding genes from *Drosophila melanogaster* (Hori *et al.*, 1985; Fouts *et al.*, 1988), human (Persico *et al.*, 1986; Chen *et al.*, 1991), rat (Ho *et al.*, 1988) *Saccharomyces cerevisiae* (Nogae and Johnstone, 1990; Thomas *et al.*, 1991), *Aspergillus niger* (van den Broek *et al.*, 1995) and *Aspergillus nidulans* (van den Broek, 1997) have been isolated and characterised. Despite the cloning and characterisation of these genes, little information is available on the regulation of their expression. It is generally accepted that G6PD expression is constitutive (Levy, 1979), which however does not exclude the possibility of changes in expression level in response to altered growth conditions.

G6PD catalyses the oxidative conversion of glucose-6-phosphate (G6P) into 6-phosphoglucono-d-lactone. This reaction is the first and committing step of the pentose phosphate pathway (Beutler and Kuhl, 1986).

The two oxidative reactions in the pentose phosphate pathway generate NADPH and the non-oxidative branch produces pentoses. In the biosynthesis of pentoses the oxidative branch is superfluous, because pentoses can also be derived from glycolytic intermediates by reversal of the transketolase and transaldolase reactions (Bonsignore *et al.*, 1974; Peleato *et al.*, 1991). Therefore, NADPH can be regarded as the most important product of the pentose phosphate pathway. But G6PD null mutants of *Escherichia coli* (Fraenkel and Vinopal, 1973) and *D. melanogaster* (Gvozdev *et al.*, 1978) do not have an apparent phenotype, which suggests that in these organisms the pentose phosphate pathway is not essential for NADPH generation.

Two growth conditions, which clearly influence the NADPH/$NADP^+$ ratio in fungi have been described: the first is growth on L-arabinose or D-xylose and the second growth on nitrate. Because in *A. niger* and *Candida utilis* the pentose reductases are NADPH specific and the pentitol oxidases NAD^+ specific, growth on L-arabinose or D-xylose requires a larger NADPH input than growth on glucose. This is reflected by a higher G6PD activity (Bruinenberg *et al.*, 1983; Witteveen *et al.*, 1987). In *A. nidulans* and *C. utilis* the same holds for growth on nitrate compared to growth on reduced nitrogen sources like ammonia or urea (Hankinson and Cove, 1974; Bruinenberg *et al.*, 1983).

The intracellular NADPH/$NADP^+$ ratio can also be influenced by drugs like paraquat, which generate superoxides. Paraquat (Methyl Viologen; 1,1'-dimethyl-4,4'bipyridinium dichloride) generates intracellular superoxides by diverting electrons from NAD(P)H to molecular oxygen (Kukielka and Cederbaum, 1990). In *E. coli*, the transcription activators *sox*R and *sox*S, which mediate the superoxide stress response, are known to increase the transcription of several genes including the G6PD encoding gene, *zwf* (Greenberg *et al.*, 1990: Liochev and Fridovich, 1992). Superoxides are detoxified by superoxide dismutase and glutathione. Following detoxification of superoxides, regeneration of glutathione involves reduction by NADPH. Therefore, drugs like paraquat consume NADPH in two ways; first NADPH acts as reductor in the generation of superoxides and at a later stage in the regeneration of glutathione. Thus, NADPH is important for the detoxification of superoxides. Because G6PD is important for the generation of NADPH, it is to be expeceted that this enzyme is also important for the detoxification of superoxides. In *S. cerevisiae*, disruption of the G6PD encoding gene *ZWF1* leads to an increased sensitivity to superoxide generating compounds (Nogae and Johnstone, 1990). Besides an increased sensitivity to superoxide generating compounds does a *S. cerevisiae ZWF1* null mutant show an absolute requirement for organic sulphur sources like methionine or cysteine (Thomas *et al.*, 1991). The basis for this organic sulphur requirement is still ill-understood. The presence of 1 mM methionine in the growth medium reduces the steady state level of *ZWF1* mRNA in a wild type *S. cerevisiae* with 50% (Thomas *et al.*, 1991).

We would like to study the effect of changes in the NADPH/$NADP^+$ ratio on the expression of the *gsd*A gene in *A. niger* to asses G6PD's role in the generation of NADPH. *A. niger* is a useful model system for this type of research, because it can use a wide array of carbon- and nitrogen sources. Furthermore, the cloning of the *A. niger gsd*A gene, encoding G6PD, allows the regulation of its expression to be studied at the molecular level. A combination of Northern blot analysis and G6PD enzyme assays should detect any change in *gsd*A expression during growth of *A. niger* on different carbon and nitrogen sources or the addition of methionine and paraquat to the growth medium.

The structure of the *A. niger* and *A. nidulans gsd*A genes is very similar: the homology at the amino acid level is 92% and positions of their nine introns are identical. In the promoter regions of the *Aspergillus gsd*A genes the overall homology is only 52%, nevertheless the *A. nidulans gsd*A gene is expressed in

A. niger transformants (van den Broek *et al.*, 1995). This proves that at least the elements responsible for basal level transcription have been conserved between the two *gsd*A promoters.

Experiments with *A. niger gsd*A cotransformants showed that the presence of one additional copy of the *gsd*A gene already inhibited the growth of the fungus especially on media containing ammonia (van den Broek *et al.*, 1995). To prevent such deleterious effects as a result of potential promoter up-mutations, arising in the deletion analysis of the *A. niger gsd*A promoter, we decided to use the *E. coli lac*Z gene as a reporter gene.

To map and identify functional elements, we fused the *A. niger gsd*A promoter and deletion derivatives to the ß-galactosidase (*lac*Z) reporter gene in the pAB94-11 vector. This promoter probe vector is one of a family of three plasmids, differing in the phase of a unique *Bam*HI cloning site with respect to the *lac*Z open reading frame (van Gorcom and van den Hondel, 1988). By insertion of putative promoter sequences, including a translational initiation codon, into the *Bam*HI site of the appropriate pAB vector a translational fusion with the *lac*Z coding region is created. The mutant *pyr*A allele in the pAB94 vector family, serving as fungal selection marker, allows targeting of the promoter constructs to the *A. niger pyr*A locus. This approach circumvents variation caused by differences in chromosomal environment (Hamer and Timberlake, 1987). Furthermore in this manner a high frequency of single copy integration of the promoter constructs is obtained, which simplifies the interpretation of the results.

Performing the promoter analysis under the aforementioned growth conditions, would allow localisation of the cis-acting elements involved in the regulation of the *A. niger gsd*A gene.

Materials and methods

All chemicals used were analytical grade whenever commercially available. Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) was obtained from Sigma. Radiochemicals were purchased from Amersham.

Strains, plasmids and growth conditions

For transformation and propagation of plasmids *E. coli* DH5α (λ⁻, F⁻, *end*A1, *hsd*R17 (r_k^- m_k^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, ϕ80Δ*lac*ZM15 was used (Focus, 1986: Sambrook *et al.*, 1989). *A. niger* N402 *csp*A1 (Bos *et al.*, 1988) was used for expression studies and *A. niger* N593 *csp*A1, *pyr*A6 (Goosen *et al.*, 1985) was used as recipient for the promoter probe constructs. Transformation of *A. niger* was performed as described previously (van den Broek *et al.*, 1995). MM (composed of 1.5 g/l KH_2PO_4 (pH 6.0), 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl) was sterilised by autoclaving. All other medium components were filter sterilised. The MM was supplemented with 0.01% yeast extract to aid germination. The carbon sources D-glucose, or D-xylose were added to a final concentration of 50 mM. Urea (Ultrapure BRL) was used in a final concentration of 5 mM, and L-proline, NH_4Cl and $NaNO_3$ in a final concentration of 10 mM. Methionine was used in a final concentration of 1mM. Approximately 10^7 conidiospores were used to inoculate 100 ml of minimal medium in 250 ml conical flasks. The cultures were incubated for 21 hours at 30°C in a New Brunswick orbital shaker at 250 rpm.

The plasmids pUC19 (Yanish-Perron *et al.*, 1985) and pTZ18 (USB) were used as general cloning vectors. The vector pAB94-11 (van Gorcom and van den Hondel, 1988) was used as promoter probe vector in this study. The plasmid pGW255 was constructed by cloning the 4 kb *Eco*RI fragment from

pGW252 (van den Broek *et al.*, 1995) into pTZ18 (USB). The plasmid pAB5-1, containing the *A. niger* glyceraldehyde-3-phosphate dehydrogenase encoding gene *gpd*A (R.F.M. van Gorcom, unpublished), was used as an internal standard in hybridisation studies.

Isolation and manipulation of nucleic acids

General manipulations of nucleic acids were performed according to Sambrook *et al.* (1989). Dideoxy-sequencing (Sanger *et al.*, 1977) was performed on CsCl gradient purified ds-plasmids using Taq DNA polymerase sequencing kits (Promega) according to the protocols from the supplier. Gel purified restriction fragments were labelled by the random primed labelling method (Feinberg and Vogelstein, 1983) as described by Sambrook *et al.* (1989). DNA was isolated from mycelium as described by van den Broek *et al.* (1995). RNA for Northern blotting was isolated as described by van Kan (1991).

Computer analysis

Computer assisted analysis of the DNA sequences was done using the GCG 7.0 software package (Devereux *et al.*, 1984) on a VAX computer.

Preparation of mycelial protein extracts

Mycelium was harvested by filtration over cheesecloth, rinsed with ice cold water, blotted dry and frozen in liquid nitrogen. Immediately, 0.25 to 0.5 g mycelium was ground to powder in a micro dismembrator II (Braun FRG). The powdered mycelium was transferred to capped 12 ml polypropylene tubes and 2 ml of extraction buffer (50 mM sodium phosphate buffer (pH 7.0), 1mM EDTA and 20 mM PMSF) were added. The proteins were extracted from the mycelium for two hours in a rotary incubator at 4°C. Subsequently the tubes were spun for 10 min at 3500 rpm in a tabletop centrifuge and the upper 1 ml of the supernatant was transferred to an eppendorf tube. These eppendorf tubes were spun for 15 min at 15000 rpm in a centrifuge at 4°C and the upper 0.5 ml was transferred to a fresh tube and used for the enzyme assays.

G6PD and ß-galactosidase activity assays

ß-galactosidase activities were measured as described by Miller (1978) with ortho-nitrophenyl-ß-D-galactopyranoside (ONPG) as substrate. To screen for ß-galactosidase expression in *A. niger* transformants, conidiospores were streaked onto sterile paper filters and incubated at 30°C for 21 hours on solid MM in petridishes. Together with the filters, the adhering mycelium was removed from the plates. Subsequently the mycelium was rendered permeable by freezing in liquid nitrogen and thawing at room temperature (Kolar *et al.*, 1991). The filters were incubated at 37°C in 5 ml of 50 mM sodium phosphate (pH 7.0), 1 mM EDTA, 20 mM PMSF and 1 mg/ml 5-bromo-4-chloro-3-indoyl-ß-galactopyranoside (X-gal). G6PD activity in protein extracts was assayed according to Löhr and Waller (1974). Protein concentrations were determined according to Lowry (1951) using the BioRAD kit. BSA (fraction V, Boehringer Mannheim) was used as a standard. The statistical significance of observed differences was tested by a two-tail Students' T-test.

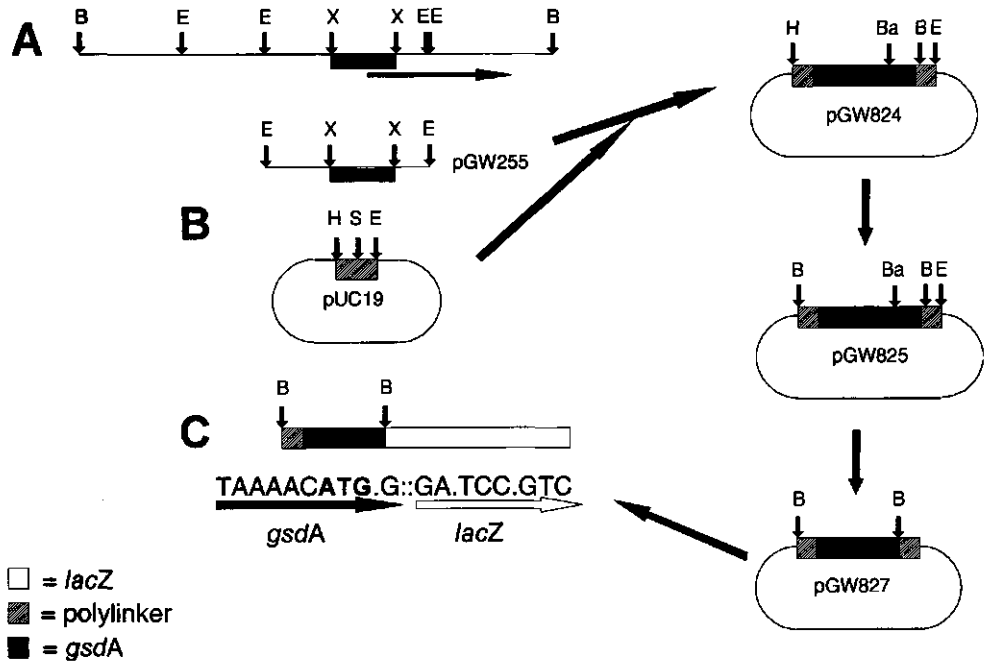

Figure 1. Construction of pGW827.

Restriction sites: B=*Bam*HI, Ba=*Bal*I, E=*Eco*RI, H=*Hind*III and X=*Xho*I. **Panel A:** Restriction map of the *A. niger gsd*A gene. The arrow marks transcribed region and the 1.3 kb *Xho*I fragment is indicated with a black box. **Panel B: The** 1.3 kb *Xho*I fragment was isolated from *gsd*A subclone pGW255 and cloned into the *Sal*I site of pUC19 to give pGW824. In pGW824 the *Hind*III site of the pUC19 polylinker was changed into a *Bam*HI site to create pGW825. To construct pGW827, all of the *gsd*A coding region downstream of the ATG was removed by deleting the smallest *Bal*I-*Bam*HI fragment from pGW825. **C:** By cloning the *Bam*HI insert of pGW827 into the unique *Bam*HI site of pAB94-11 a translational fusion was created between the *gsd*A ATG and the coding region of the *E. coli lac*Z gene. The *gsd*A ATG is in bold print and the *lac*Z open reading frame is indicated by interpunction of the codons.

Blotting and hybridisation

All solutions for Northern blotting were prepared in diethylpyrocarbonate (DEPC) treated water. Total RNA was dissolved in 40 mM MOPS (pH 7.0), 10 mM NaAc, 1 mM EDTA, 2.2 M formaldehyde and 50% formamide, heat denatured for 5 min at 65°C and cooled on ice. The samples were applied to an 1.5% agarose gel containing 40 mM MOPS pH (7.0), 2.2 M formaldehyde, 10 mM NaAc and 1 mM EDTA and electrophoresed in the same buffer without formaldehyde. The RNA was blotted in 10xSSC onto HybondN$^+$, crosslinked by 5 min UV irradiation (254 nm) on a transilluminator, prehybridised for three hours at 42°C and hybridised according to the manufacturer's instructions. Dot blotting and Southern analysis were performed with HybondN$^+$ according to the alkaline blotting protocol supplied by Amersham. Densitometry of autoradiographs was performed with a Cybertech CS-1 CCD camera and software (Dalton).

Results

Construction of an A. niger gsdA promoter-E. coli lacZ fusion.

To construct the gsdA promoter-E. coli lacZ fusion, the 1.8 kb XhoI fragment, comprising the N-terminal part of the coding region and 5' proximal sequences, was isolated from gsdA subclone pGW255 (Fig. 1B). This 1.8 kb XhoI fragment was cloned into the SalI site of pUC19. By cloning a linker into the HindIII site of the pUC19 polylinker, a BamHI site was added to the 5' end of the gsdA promoter region. At the 3' end of the promoter fragment, another BamHI site was introduced immediately downstream of the putative gsdA initiation codon by deleting the 0.8 kb BalI-BamHI fragment, which concomitantly deleted all of the gsdA coding region downstream of the ATG (Fig. 1C).

By cloning the resulting *Bam*HI fragment into the unique *Bam*HI site of pAB94-11 a translational fusion was created between the putative *gsd*A ATG and the *lac*Z coding region. This plasmid was named pGW828. Since pAB94-11 also contains a mutant *A. niger pyr*A selection marker, pGW828 could be used to transform *A. niger* N593 to uracil prototrophy. The transformants thus obtained, were tested for their ability to hydrolyse X-Gal. In this experiment an *A. niger trp*C-null mutant, carrying an in frame *lac*Z gene insertion in the *trp*C coding region (Goosen *et al.*, 1988), served as a positive control. Transformants with a single copy of pAB94-11 were used as a negative control. Approximately half of the transformants obtained with pGW828 stain blue, which proves that 990 bp upstream of the translational initiation codon are sufficient for *gsd*A promoter activity. Throughout this study, pGW828 will be considered to contain a full length *gsd*A promoter.

By Southern analysis two transformants, carrying a single copy of pGW828 integrated at the *pyr*A locus, were selected and these transformants were named D0.

Analysis of steady state *gsd*A mRNA levels and G6PD enzyme activity under different physiological conditions

To gain insight into the regulation of *gsd*A expression, steady state mRNA levels as well as G6PD enzyme activities were studied under a number of physiological conditions. These conditions were selected because they are known to affect NADPH consumption in Aspergillus and/or other organisms. The analysis was performed both in the *A. niger* wild type strain N402 and transformant D0, to detect any interference between expression of the *gsd*A promoter-*lac*Z fusion and the wild type *gsd*A gene.

Total RNA was extracted from transformant D0, grown for 21 hours on glucose/urea, glucose/urea/NO_3^-, glucose/urea/NO_3^-/NH_4^+ and glucose/proline. Northern blots of this RNA were probed with the 1.8 kb *Nco*I fragment of the *A. niger gsd*A gene, that encompasses most of its coding region (van den Broek *et al.*, 1995). In all Northern blot experiments the 1.6 kb *Xho*I fragment of the *A. niger* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*A), comprising most of the coding region (R.F.M. van Gorcom, pers. comm.), was used as an internal standard. In all experiments the hybridisation signals obtained with the *gpd*A probe (fig. 2, panel B) were at least fifty fold stronger than with the *gsd*A probe.

Although some variation was found, no systematic change was observed in the steady state *gsd*A mRNA levels during growth on urea/NO_3^- and urea/NO_3^-/NH_4^+, compared to growth on urea alone (fig. 2). This indicates that the *A. niger gsd*A expression is neither subject to NO_3^- induction nor nitrogen metabolite repression. Also on glucose/proline *gsd*A expression equals expression on glucose/urea. Furthermore, in protein extracts from transformants grown on the same medium, no change in G6PD enzyme activity was observed under these conditions. Identical results were obtained with the *A. niger* N402 wild type strain, both in Northern blot experiments and in G6PD enzyme assays (not shown).

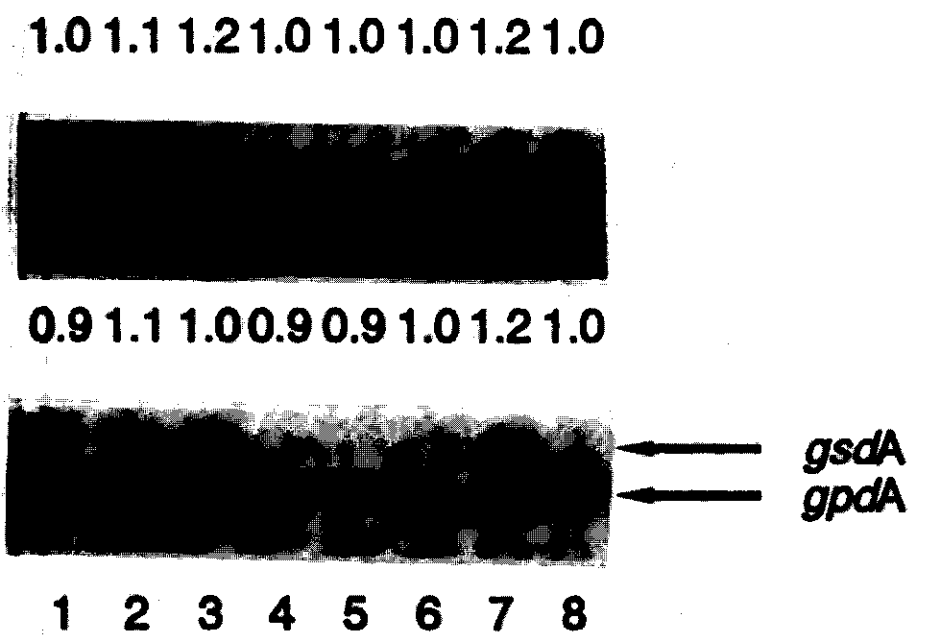

Figure 2. Northern blot analysis of *gsd*A expression.

Total RNA was isolated from transformant D0, which was grown for 21 hours in 100 ml MM medium with different carbon sources (50 mM) and nitrogen sources (5 mM), 10 mM methionine or 1 mM paraquat. Approximately 50 µg total RNA was separated on an agarose gel, blotted and hybridised with the 1.8 kb *Nco*I fragment of the *A. niger gsd*A gene (upper panel). To correct for differences in the amount of RNA loaded, the blots were rehybridised with the 1.6 kb *Xho*I fragment of the *A. niger gpd*A gene (lower panel). Upper panel: Northern blot probed with the 1.8 kb *Nco*I *gsd*A probe, exposure time 16 hours, lane 1: glucose/urea, lane 2: glucose/urea/NO_3^-, lane 3: glucose/urea/NO_3^-/NH_4^+, lane 4: glucose/proline, lane 5: glucose/urea/methionine, lane 6: glucose/urea/paraquat, lane 7: xylose/urea, lane 8: glucose/xylose/urea. Under each of these growth conditions G6PD activity in protein extracts was monitored directly as described by Löhr and Waller (1974) and the values obtained are indicated between the upper and lower panel in µmoles NADPH/min/mg total protein. Lower panel: Without removal of the *gsd*A probe the blot was rehybridised with the 1.6 kb *Xho*I *gpd*A probe. The ratio between the *gsd*A hybridisation signal on glucose/urea and a particular growth condition is indicated above each lane on the upper panel.

To determine the possible effect of superoxides on *gsd*A mRNA levels, both *A. niger* N402 and transformant D0 were exposed to the superoxide generating compound paraquat. First the sensitivity of *A. niger* to this compound had to be determined. On solid minimal medium, exposure to 10 mM paraquat reduces colony size with 50% and a concentration of 40 mM paraquat is required to inhibit growth completely (not shown). However in liquid MM (glucose/urea), supplemented with 10 mM paraquat no growth occurs. The presence of 1 mM paraquat also inhibits growth of transformant D0, but sufficient biomass is formed to allow RNA extraction. After 21 hours of incubation, no change in *gsd*A expression was detected at the mRNA level (fig. 2: panel A, lane 6). Addition of 1 mM paraquat to an overnight culture of transformant D0, followed by four hours of incubation, did not result in an increase in *gsd*A transcript either. Again in *A. niger* N402 identical results were obtained.

Addition of 1 mM methionine to the growth medium did not reduce *gsd*A mRNA levels in transformant

D0 or N402 as was reported for the *S. cerevisiae ZWF1* gene (Thomas *et al.*, 1991). There was neither an effect of methionine on the G6PD enzyme activity in D0 nor in the wild type N402 (not shown). No significant increase in *gsd*A/*gpd*A mRNA ratio was observed with D-xylose as carbon source in D0 and *A. niger* N402. Also on a mixture of D-glucose and D-xylose the steady state level of *gsd*A messenger is identical to the level on D-glucose alone (fig. 2). Both in transformant D0 and in *A. niger* N402, no consistent change was observed in G6PD enzyme activity during growth on D-xylose.

Under all conditions tested, identical results were obtained with the wild-type *A. niger* strain N402 and transformant D0. This indicates that the presence of an additional copy of the *gsd*A promoter does not interfere with expression of the resident *gsd*A gene.

Sequence comparison of the 5' proximal regions of the *Aspergillus gsd*A genes

Comparison of the 5' proximal regions of homologous genes of filamentous fungi has been shown to be a valuable tool to select target sequences for promoter analysis (Punt *et al.*, 1989; Punt and van den Hondel, 1992). Conserved sequence motifs, thus identified, can be mutagenised *in vitro* and the resulting mutations can be tested for their effect on promoter activity *in vivo*.

To identify conserved sequence elements in the *A. niger* and *A. nidulans gsd*A promoters, the sequences between -1000 and the ATG (+1) were aligned according to the algorithm of Needleman and Wunsch (1970). Four short stretches of strong sequence conservation, 11 to 22 bp long, are observed in the region between -325 and +1. The order of these four sequences is identical for both *Aspergillus gsd*A promoters, but their mutual spacing varies slightly (Fig. 3). A search of the GENBANK/EMBL database produced no sequences related to these four sequence motifs in the 5'proximal regions of other genes from filamentous fungi. And although their role in *gsd*A transcription has not yet been established, we tentatively named them *gsd*A boxes 1, 2, 3 and 4. Downstream of the *gsd*A boxes both *Aspergillus gsd*A promoters contain several C+T rich stretches (CT boxes). The *A. nidulans gsd*A promoter has shorter CT boxes and a larger number of transcription startpoints (tsps) compared to *A. niger* (van den Broek *et al.*, 1995). The four transcription starts in the *A. niger gsd*A promoter are localised in a CT box, downstream of *gsd*A box 4. In the *A. nidulans gsd*A promoter, the eight tsps are distributed over an area that begins downstream of *gsd*A box 2 and ends just downstream of *gsd*A box 4. Upstream of -325 the homology in the Aspergillus *gsd*A promoters is less obvious, yet short stretches of sequence conservation are present throughout the 5' proximal region.

Aligning the *A. niger gsd*A and *S. cerevisiae ZWF1* promoters, sequences exhibiting 50 to 70% sequence identity with *gsd*A boxes 1, 2 and 4 were discovered (fig. 4). No Aspergillus *gsd*A box 3 homologue was found in the *S. cerevisiae ZWF1* promoter. Like in both *Aspergilli* the order of the *gsd*A boxes, but not their mutual spacing, has been conserved in *S. cerevisiae*.
The two tsps in the *S. cerevisiae ZWF1* gene map in a 63 bp C+T rich sequence (Nogae and Johnstone, 1990) approximately 130 bp downstream of *gsd*A box 4 homologue. Between the *gsd*A box 4 homologue and the tsps also two putative TATA boxes are present.

Construction of *gsd*A promoter deletion mutations

Based on the information from the alignment, the deletions in the *gsd*A promoter were designed. Using internal restriction sites, we introduced eleven deletions into the 1020 bp *Bam*HI insert of pGW827

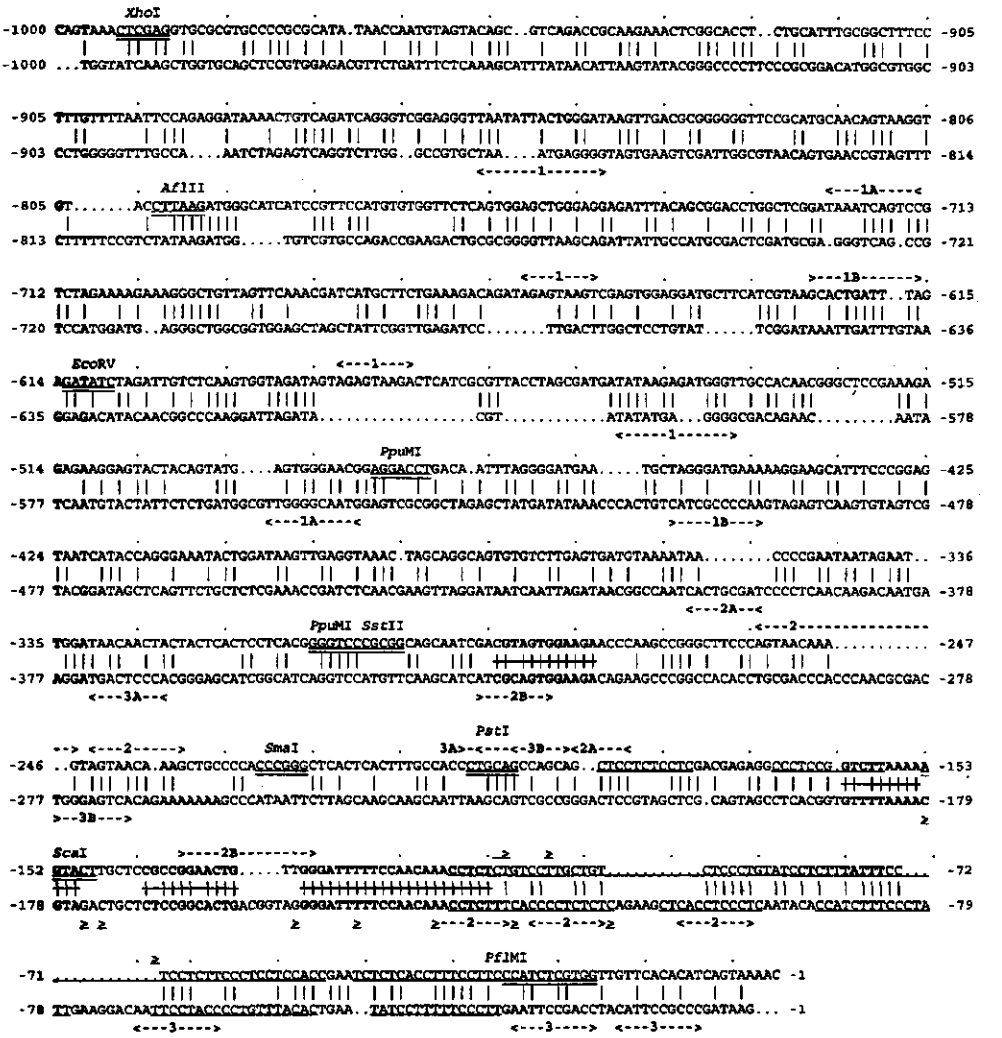

Figure 3. Alignment of the *A. niger* and *A. nidulans gsd*A promoters.

The *A. niger* (upper) and *A. nidulans* (lower) *gsd*A 5' proximal regions were aligned according to the algorithm of Needleman and Wunsch (1970). In both sequences, which are numbered with respect to the putative initiation codon (A in ATG = +1), the CT boxes are underlined and the transcription start points (tsp) are indicated with ≥ signs. The inverted (<--< or >-->) and direct repeats (<-->) in the *A. niger* promoter are marked by dashed arrows above the *A. niger* sequence and in the *A. nidulans* promoter below the sequence. The restriction sites, used to construct the promoter deletions, are indicated above the *A. niger gsd*A promoter sequence. The four *gsd*A boxes are marked by a line through the bars that indicate a basepair match (|||||).

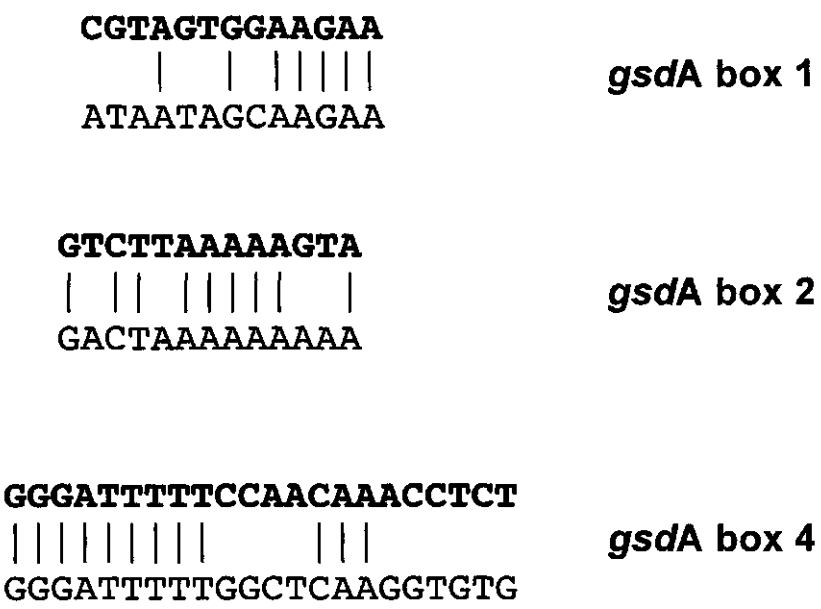

Figure 4. Aspergillus *gsd*A box homologues in the *S. cerevisiae ZWF1* promoter.
Sequence motifs that are 50-70% homologous to the Aspergillus *gsd*A boxes 1, 2 and 4, are found beween -270 and -33 (ATG=+1) in the *S. cerevisiae ZWF1* promoter. The upper sequence represents the *A. niger gsd*A boxes in bold print and the lower sequence the *S. cerevisiae* homologues. Immediately upstream of the *gsd*A box 1 homology a putative second open reading frame is found, that could limit the length of the *ZWF1* promoter to 350 bp (Johnstone and Nogae, 1990).

(Fig. 5, D1 to D10). The presence of the desired deletions in the constructs was checked by restriction analysis with *Taq*I (not shown).

The deletion derivatives of the *gsd*A promoter were cloned into the unique *Bam*HI site of pAB94-11. After the orientation of the *Bam*HI insert in pAB94-11 had been established, all constructs were introduced into *A. niger* N593. Using dot blotting and Southern analysis the Aspergillus transformants were checked for single copy integration of the promoter probe constructs at the *pyr*A locus. Transformants containing a single copy of pAB94-11 served as a promoterless control. For every construct two independent transformants were selected for the ß-galactosidase assays.

Deletion analysis of the *A. niger gsd*A promoter

To analyse *gsd*A promoter activity transformants D0 to D10 and the background control pAB94-11 were grown for 21 hours on glucose/urea. Protein extracts of all transformants were assayed for ß-galactosidase activity.

On this medium, deletions D3, D4, D9 and D10 reduce ß-galactosidase activity to background level, but deletions D1, D2, D5, D6, D7 and D8 retain considerable transcription (table 1). This identifies the sequences downstream of the *Sst*II site (-298) as essential for *gsd*A transcription. This essential region contains all four *gsd*A boxes and both CT boxes. This suggests that these boxes are functionally

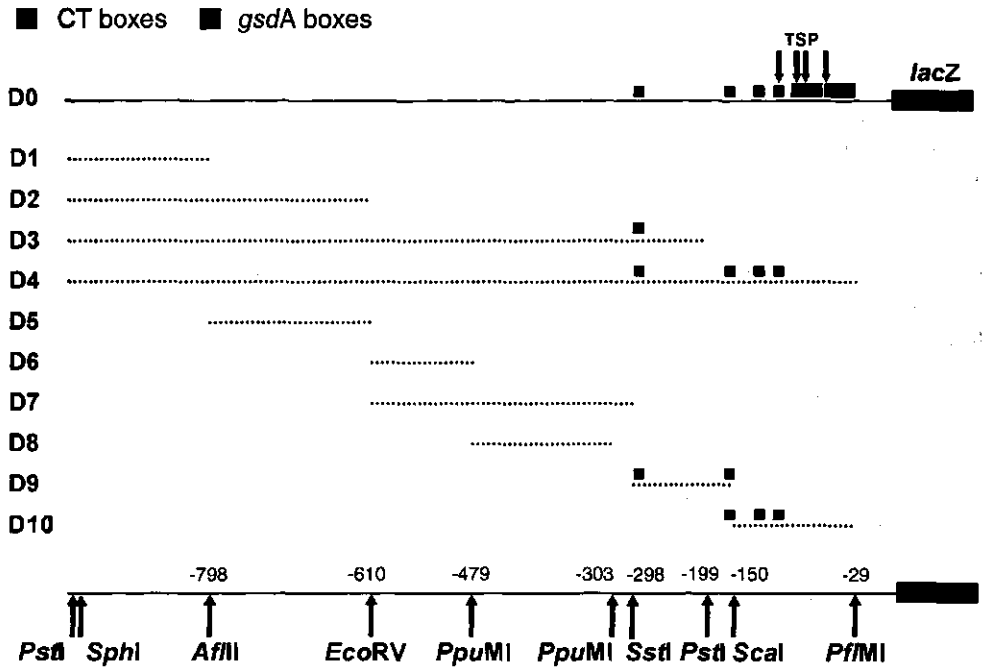

Figure 5. Deletions in the *A. niger gsd*A promoter.

For the construction of deletion derivatives of pGW827 only restriction sites internal to the 1020 bp *Bam*HI insert were used. In general pGW827 was digested with both enzymes, gel purified and religated. If necessary, protruding ends were either filled in or removed with T4 DNA polymerase. Because pGW827 contained multiple *Ppu*MI and *Sca*I sites, the plasmid had to be partially digested with *Ppu*MI for the construction of D6 and with *Sca*I for the construction of D9 and D10. Subsequently the linearised plasmid was digested with the second enzyme, blunt ended and religated. The presence of the desired deletions was checked by restriction analysis with *Taq*I. The 1020 bp long *Bam*HI fragment of pGW827 is depicted as D0 and is considered to comprise the full *A. niger gsd*A promoter. The restriction sites, used to construct the deletions are indicated with arrows in the bottom part of the picture. The sequences deleted in D1 to D10 are indicated with a dashed line. The *gsd*A boxes 1, 2, 3 and 4 are depicted as black boxes, the CT boxes as hatched boxes and the transcription start points (tsp) are marked with arrows. The distance of the restriction sites relative to the ATG (+1) is also indicated. The *Pst*I and *Sph*I site, at the 5' proximal end of D0, reside in the 19 bp long sequence derived from the pUC19 polylinker.

important for *gsd*A transcription and therefore might constitute a minimal *gsd*A promoter (fig. 5).

On glucose/urea, the ß-galactosidase activity in transformant D0 is 13.5 nmol 2-nitrophenol/min/mg protein (table 1). This is in the same order of magnitude as the activity of the *A. nidulans trp*C promoter-*lac*Z fusion (Hamer and Timberlake, 1987), but 100-fold lower than the *A. nidulans gpd*A promoter-*lac*Z fusion (Punt *et al.*, 1988). This confirms our conclusion from the Northern blot analysis and indicates that the *A. niger gsd*A promoter is a weak promoter.

Deletions that map upstream of the *Sst*II site, where the *A. niger* and *A. nidulans* promoter sequences diverge, are also found to have a significant effect on the expression of the *lac*Z gene (fig. 5). To investigate these transcription modulating sequences in more detail, all transformants were grown for 21 hours under conditions reported to influence G6PD expression (Hankinson and Cove, 1974; Wit-

Table 1. β-galactosidase activity of *gsd*A promoter-*E. coli lac*Z fusions.

Specific ß-galactosidase activity in *A. niger* transformants D0 to D10 expressed as nmoles 2-nitrophenol/min/mg total protein, measured after 21 hours of growth under the following conditions glucose (50 mM)/urea (5 mM), glucose/urea/nitrate (10 mM), glucose/urea/nitrate/ammonia (10 mM), glucose/urea/methionine (1 mM), D-xylose (50 mM)/urea, D-xylose (50 mM)/glucose (50 mM)/urea. The number of independent experiments is indicated above each column (N=). However, the data in the rows marked with an asterisk (*) are the mean of only two independent experiments. For each construct and each condition, the standard deviation is indicated between brackets. **ND** = not determined. The ß-galactosidase activities marked with ' differ significantly (at 10% level in a two tailed Student's T-test) from the activity of construct D0. Transformant pAB94-11 contains a promoterless reporter construct, that serves as a background control.

	Glucose Urea (N=8)	**Glucose Urea NO_3 (N=10)**	**Glucose Urea NO_3/NH_4^+ (N=3)**	**Glucose Urea Methionine (N=4)**	**Xylose Urea (N=3)**	**Gluxose Xylose Urea (N=2)**
D0	13.5	13.1	11.0	11.6	7.0	14.2
	(3.6)	(3.7)	(1.5)	(4.5)	(1.5)	(3.7)
D1	10.0'	9.2'	9.0'	9.5	5.7	10.1
	(3.6)	(2.4)	(0.3)	(4.3)	(1.0)	(3.8)
D2	14.6	14.8	17.3'	14.3	6.7	13.3
	(3.2)	(4.5)	(2.8)	(7.1)	(0.5)	(1.6)
D3*	0.5'	0.1'	ND	0.5'	ND	ND
	(0.4)	(0.1)		(0.3)		
D4*	0.3'	0.2'	ND	0.3'	ND	ND
	(0.2)	(0.1)		(0.1)		
D5	11.8	12.2	15.0	12.0	6.3	9.2
	(2.5)	(4.0)	(3.0)	(7.1)	(0.8)	(1.5)
D6	10.7'	10.0'	11.4	10.0	5.7	10.4
	(2.3)	(2.8)	(1.2)	(4.5)	(1.2)	(3.0)
D7	8.1'	7.5'	4.8'	6.6	4.2'	7.1'
	(2.4)	(2.1)	(1.4)	(3.1)	(1.0)	(2.7)
D8	11.8'	11.7	7.9'	11.0	6.4	12.0
	(3.0)	(4.7)	(1.9)	(4.2)	(1.6)	(3.3)
D9*	0.3'	0.1'	ND	0.3'	ND	ND
	(0.3)	(0.1)		(0.3)		
D10*	0.2'	0.1'	ND	0.2'	ND	ND
	(0.1)	(0.1)		(0.1)		
pAB94/11	0.3'	0.2'	0.1'	0.3'	0.1'	0.1'
	(0.3)	(0.1)	(0.1)	(0.3)	(0.1)	(0.1)

teveen *et al.*, 1987) and assayed for both *lac*Z (table 1) and G6PD enzyme activity. No significant deviation of the G6PD enzyme activity was detected in any of the transformants under any of the growth conditions compared to the pAB94-11 transformant or the wild type (data not shown). This again shows the absence of interference of the promoter-*lac*Z fusion constructs and the resident *gsd*A promoter.

On all media, deletion of the region between -991 and -798 (D1) decreases *lac*Z expression by 20%, while further deletion to -610 (D2) restores the activity to a level even higher than D0. This indicates that a negative regulating factor binds between the -798 and -610. The effect of deletion D2 is most pronounced on glucose/urea/NO_3^-/NH_4^+. However, specific deletion of the -798/-610 region (D5) does not cause a significant increase in ß-galactosidase activity on any of the media.This observation contradicts the presence of a repressor acting on the -798/-610 region.

Deletion of the -610/-479 region (D6) does not influence ß-galactosidase activity on glucose/urea/NO_3^-/NH_4^+, but on all other media it causes a decrease. On glucose/urea/NO_3^-/NH_4^+ deletions D2, D5 and D6 become slightly more active than on glucose/urea or glucose/urea/NO_3^-, despite the fact that the absolute promoter activity in D0 compared to glucose/urea or glucose/urea/NO_3^- does not change. The physiological significance of this phenomenon is unclear.

Under all growth conditions tested complete deletion of the -610/-303 region (D7) affects ß-galactosidase activity more than the sum of the effects of deleting the -610/-479 (D6) and -479/-298 (D8) regions. This could either point towards a putative transcription factor that binds between -303 and -298 or a cooperative effect on transcription by the putative trans-acting factors, that bind in D6 and D8.

Discussion

With Northern blot analysis, G6PD activity assays and promoter-*lac*Z fusions, we studied the regulation of the *A. niger gsd*A promoter and its functional organisation. A similar analysis was used to study the *A. nidulans trp*C (Hamer and Timberlake, 1987), the *A. nidulans gpd*A (Punt *et al.*, 1990), the *A. nidulans aba*A (Adams and Timberlake, 1991), the *A. niger* (Fowler *et al.*, 1990) and *A. oryzae gla*A (Hata *et al.*, 1992) promoters.

The observed differences in the ß-galactosidase activity of the *gsd*A promoter deletions indicate that the *A. niger gsd*A promoter contains sequences that interact with positive or negative regulating trans-acting factors. However, deletions as such can influence promoter activity by altering the spacing of promoter elements or nucleosomes (McKnight and Kingsbury, 1982). Even new promoter elements could be created by ligation of the deletion end-points (Breathnach and Chambon, 1981).

We found that transcription of the *A. niger gsd*A gene, steady state *gsd*A mRNA levels or G6PD enzyme activity does not increase in response to medium components, that are reported to increase NADPH demand (Hankinson and Cove, 1974, Witteveen *et al.*, 1987). The latter suggests that the two oxidative reactions of the pentose phosphate pathway can neither be the sole nor primary sources of NADPH in *A. niger*. Other potential sources for NADPH are the reactions catalysed by NADP-linked isocitrate dehydrogenase and NADP-linked malate dehydrogenase. These enzymes have not been studied in *A. niger*, but in *A. nidulans* both have been shown to make only a minor contribution to NADPH production on glucose (Singh *et al.*, 1988). In the fungus *Alternaria alternata* NADPH is reported to be generated by a cyclical interconversion of mannitol into fructose-6-phosphate, in which reduction equivalents are shuttled from NADH to $NADP^+$ at the expense of ATP (Hult and Gatenbeck, 1978). The presence of the enzymes involved in this mannitol cycle have been demonstrated in *A. niger* and several other *fungi imperfecti* (Hult *et al.*, 1980). However, the presence of the relevant enzyme activities in

a fungus does not necessarily imply the operation of the mannitol shuttle. For example in *A. nidulans* all the enzymes for the operation of the mannitol shuttle are present, but a coordinated increase of these enzymatic activities in response to an increased NADPH demand could not be demonstrated (Singh *et al.*, 1988).

Both *A. nidulans* pentose phosphate pathway mutants *ppp*A and *ppp*B grow poorly on nitrate as sole nitrogen source (Hankinson, 1974). This suggests that in *A. nidulans* the two oxidative reactions in the pentose phosphate pathway are the primary source for NADPH. This would imply that the regulation of *gsd*A expression in *A. nidulans* is different from *A. niger*. Different regulation of homologous genes in *Aspergilli* is not unprecedented, since different regulation was also reported for the aldehyde dehydrogenase genes (*ald*A) from *A. niger* and *A. nidulans* (O'Connell and Kelly, 1988). The latter implies, that in the absence of information on the regulation of gene expression, sequence comparison of the two *Aspergillus gsd*A promoters as a tool to identify cis-acting elements is unreliable. Still, four strongly conserved sequence motifs, the putative *gsd*A boxes, were identified in this way. The region in which the *gsd*A boxes reside is essential for transcription, which is consistent with their conservation in two differently regulated Aspergillus genes. The region indispensable for transcription in the *A. niger gsd*A promoter is unusually large, for in the *A. nidulans arg*B (Goc and Wegelenski, 1988), *gpd*A (Punt *et al.*, 1990) and *trp*C (Hamer and Timberlake, 1987) promoters 50 bp upstream of the transcription start is sufficient to drive transcription. In this respect the *A. niger gsd*A promoter bears more resemblance to the *A. nidulans pgk*A promoter, where the region between 161 and 120 bp upstream of the transcription start proved to be vital for promoter function (Streadfield *et al.*, 1992). Since the spacing of the four *gsd*A boxes is different in the two Aspergillus promoters, the four *gsd*A boxes represent possibly separate cis-acting elements. Also the fact that not all four *gsd*A boxes have been conserved in the *S. cerevisiae ZWF1* promoter, indicates that they could function as independent promoter elements. In general both promoter elements and splicing signals from filamentous fungi are distinct from expression and splicing signals in *S. cerevisiae* (Punt and van den Hondel, 1992: Unkles, 1992) and this often prevents expression of genes from filamentous fungi in yeast (Rambosek and Leach, 1987). By contrast, the G6PD encoding genes from these species share both three out of four *gsd*A boxes as well as C+T rich stretches in their 5' proximal sequences. In the *A. nidulans gpd*A and *oli*C promoters C+T rich sequences, but not TATA boxes, have been shown to determine the site of transcription initiation (Punt *et al.*, 1990: Punt and van den Hondel, 1992). Since both *ZWF1* transcription start points are mapped to the first purine downstream of a C+T rich sequence, this sequence could represent a functional *S. cerevisiae* CT box. On the other hand, there are also two putative TATA boxes, that often determine transcription initiation in *S. cerevisiae*, upstream of both tsps in *ZWF1*. Therefore, it would be very interesting to see if the Aspergillus *gsd*A promoter would function in *S. cerevisiae* or even whether the *gsd*A promoter fused to a full length *A. niger gsd*A cDNA clone would complement the *zwf1* null mutant.
Despite some structural similarities, *A. niger gsd*A and *S. cerevisiae ZWF1* behave differently; even when it resides on a plasmid attaining 100 to 500 copies per cell, the *S. cerevisiae ZWF1* gene does not raise G6PD activity (Nogae and Johnstone, 1990), whereas for *A. niger* an almost linear response was found between the number of *gsd*A copies and G6PD activity (van den Broek *et al.*, 1995). A functional analysis of the *S. cerevisiae ZWF1* promoter is needed to show the role of the *gsd*A, the TATA, putative CT boxes and to localise the methionine responsive element.

Although D-xylose utilisation requires more NADPH than glucose (Witteveen *et al.*, 1990), no increase in G6PD enzyme activity is observed in *A. niger* during growth on D-xylose compared to growth on glucose. These results are in contrast with the results of Witteveen *et al.* for *A. niger* (1990), but are in agreement with the results obtained with *A. nidulans* (Singh *et al.*, 1988). Since G6PD enzyme activity in *E. coli* (Wolf *et al.*, 1979) and in *A. nidulans* (Carter and Bull, 1969) as well as transcription of

the G6PD encoding gene *zwf* in *E. coli* have been shown to be positively correlated with growth rate (Rowley *et al.*, 1991), the absence of an increase in G6PD activity on D-xylose could be the result of a compensatory decrease in growth rate. The latter is consistent with the observation that the biomass yield on D-xylose is lower than on glucose (PB unpub. res.).

To complicate matters further, growth on D-xylose as sole carbon source reduces the ß-galactosidase activity by 50%, whereas activity on a mixture of D-xylose and glucose is the same as on glucose alone. This decreased *gsd*A promoter activity on D-xylose compared to glucose is inconsistent with the observed G6PD enzyme activity and the *gsd*A mRNA level, which are identical under both conditions. Since this reduction in ß-galactosidase activity on D-xylose is observed in all promoter deletions tested, the elements exerting this effect should either reside within the region essential for transcription or upstream of -990. The latter would imply, that 990 bp of 5' proximal sequences do not constitute a functional *gsd*A promoter. Additionally, it can not be excluded that regulation of *gsd*A transcription at the *pyr*A locus differs from regulation at its resident locus nor that under some conditions regulation of *gsd*A expression occurs post-transcriptionally.

An alternative explanation could be the induction of a G6PD isozyme on D-xylose. The transcription of the *gsd*A gene could be lowered, while induction of such a putative *gsd*B gene would ensure stable G6PD enzyme activity. In Southern blots of chromosomal DNA from *A. niger* the presence of fragments cross- hybridising to the *gsd*A coding region were detected (van den Broek *et al.*, 1995). These fragments could be candidates to encode such a G6PD isozyme. In *Aspergillus oryzae* induction of a specific G6PD isozyme has been demonstrated during growth on L-ribose (Cébrian-Pérez *et al.*, 1989). Cébrian-Pérez and coworkers suggest, that this G6PD isozyme is induced to provide NADPH under glucose limiting conditions. Also in *A. niger* such an isozyme could be induced on D-xylose. Therefore, it would be worthwhile to isolate the putative *gsd*B gene from *A. niger* and test the hypothesis of its induction by D-xylose or L-ribose.

In any case our data provide no indication for an indispensable role of the *gsd*A gene in the generation of NADPH in *A. niger*. From genetic and biochemical data it is clear that in *A. nidulans* the two dehydrogenases of the pentose phosphate pathway play an important role in NADPH production (Hankinson, 1974: Hankinson and Cove, 1974), which makes it very interesting to perform a functional analysis of its *gsd*A promoter.

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Chapter 6 Regulation of the *Aspergillus nidulans gsd*A gene expression; Involvement of regulatory proteins of the nitrate metabolism

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Summary

In *A. nidulans* glucose-6-phosphate dehydrogenase (G6PD) activity increases in response to addition of nitrate to the growth medium. We show that this nitrate stimulation of G6PD activity is caused by increased transcription of the G6PD encoding gene (*gsd*A) and is mediated by the *nir*A gene product (NIRA). In addition to mediating the nitrate induction, NIRA is also involved in *gsd*A transcription in the absence of nitrate. Although *gsd*A expression is not subject to nitrogen metabolite repression, its mediator AREA still stimulates *gsd*A transcription. The introduction of a *gsd*A promoter-*lac*Z fusion and two deletion derivatives into wild type *A. nidulans* as well as into *nir*A1, *nia*D15, *are*A19 and *xpr*D1 mutants, allowed an approximate localisation of the NIRA and AREA sites of action. Both NIRA and AREA stimulate *gsd*A transcription from positions between 1219 and 742 bp upstream of the translational initiation codon. Our data prove that the regulation of the *A. nidulans gsd*A gene expression differs from the regulation of its *A. niger* homologue. Furthermore, in contrast to the *A. niger gsd*A promoter, deletion of the most upstream *gsd*A box in *A. nidulans* does not result in a complete loss of promoter activity.

Introduction

In glucose grown *Aspergillus nidulans* mycelium a substantial part of the NADPH, required for nitrate and nitrite reduction, is generated by the two oxidative reactions of the pentose phosphate pathway (Hankinson and Cove, 1974; Hankinson, 1974: Singh *et al.*, 1988). These reactions are catalysed by glucose-6-phosphate dehydrogenase (G6PD: ß-D-glucose-6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD: 6-phospho-D-gluconate:$NADP^+$ 2-oxidoreductase, EC 1.1.1.44). Growth on nitrate stimulates the flux of metabolites through the pentose phosphate pathway (Carter and Bull, 1969) by inducing an increase in the G6PD, 6PGD, transaldolase, transketolase and glucose-6-phosphate isomerase activities (Hankinson and Cove, 1974). So it is evident that NADPH production by the pentose phosphate pathway and NADPH consumption by the nitrate and nitrite reductases are coordinated.

In *A. nidulans* the expression of the structural genes of the nitrate utilisation pathway *nia*D, *nii*A and *crn*A (Johnstone *et al.*, 1990: Unkles *et al.*, 1991), encoding nitrate reductase, nitrite reductase and nitrate permease respectively, is subject to two control mechanisms: induction by nitrate or nitrite and re-

pression by ammonia or glutamine (Cove, 1979; Tomsett, 1989). Nitrogen sources like urea or L-proline neither induce nor repress expression of the nitrate utilisation genes (Hawker *et al.*, 1992). In *A. nidulans*, ammonia repression or, more correctly, nitrogen metabolite repression is exerted by the *are*A gene product (Arst and Cove, 1973). The *are*A gene encodes a positive-acting regulatory protein (AREA). When ammonia or glutamine are not available, AREA stimulates the expression of numerous genes required for the utilisation of other nitrogen sources. Consequently, loss of function mutations in *are*A result in an inability to utilise nitrogen sources other than ammonia or glutamine (Arst and Cove, 1973: Cove, 1979). The *are*A allele *xpr*D1 has the reverse effect and leads to derepressed expression of nearly all activities under *are*A control (Arst, 1982). Two domains in the deduced amino acid sequence of the *are*A gene suggest that the AREA protein is a transcriptional activator: an acidic domain, that forms an amphipathic α-helix, and a putative zinc finger (Caddick *et al.*, 1986; Caddick, 1992). The AREA protein is homologous to the *nit-2* gene product (NIT2), that mediates nitrogen metabolite repression in *Neurospora crassa* (Fu and Marzluf, 1990). The *N. crassa nit-2* gene product has been shown to substitute for AREA in *A. nidulans* (Davis and Hynes, 1987) and to bind to sites with the core sequence TATCTA or its complement in the *N. crassa nit-3* (nitrate reductase) promoter, the *A. nidulans nia*D promoter (Fu and Marzluf, 1990) and the *Lycopersicon esculentum nia* (nitrate reductase) promoter (Jarai *et al.*, 1992). Glutamine, the effector of nitrogen metabolite repression, is thought to prevent binding of AREA to its target sequences (Caddick, 1992).

Nitrate or nitrite induction of the nitrate utilisation pathway is mediated by the product of the regulatory gene *nir*A (See Cove, 1979; Tomsett, 1989). The role of *nir*A was deduced from mutant alleles resulting in either no induction by nitrate or nitrite (*nir*A^{-}) or constitutive (*nir*A^{c}) expression of *nia*D, *nii*A and *crn*A (Cove, 1979). *nir*A encodes a protein (NIRA) that has the distinctive characteristics of a GAL4-type transcriptional activator: a putative zinc-binding motif, an acidic domain and two proline rich regions (Burger *et al.*, 1991b). NIRA has been shown to be the limiting factor in the regulation of *nia*D and *nii*A mRNA levels (Hawker *et al.*, 1992) The strong derepressed synthesis of nitrate and nitrite reductase by the *nir*A^{cd}101 mutant in the presence of ammonia (Rand and Arst, 1978), suggests interaction or cooperation between NIRA and AREA. This cooperation does not act on the transcription of *nir*A for steady state *nir*A mRNA levels are not appreciably increased by nitrate nor repressed by ammonia or glutamine (Burger *et al.*, 1991a/b). The nitrate reductase holoenzyme might also interact directly with NIRA, because the majority *nia*D and *cnx* mutants exhibit constitutive expression of all nitrate utilisation genes which are under NIRA control (Pateman *et al.*, 1964).

A model that explains both the autoregulatory properties of *nia*D and the apparent constitutive expression of *nir*A was described by Cove (1979). According to this model, the low steady state level of NIRA leads to low level of *nia*D transcription. In the absence of nitrate, the nitrate reductase would inactivate the constitutively expressed NIRA. In the presence of nitrate however, nitrate reductase no longer would inactivate NIRA, leading to induced levels of the enzymes of the nitrate utilisation pathway.

The nitrate induced increase in G6PD activity in *A. nidulans* is mediated by NIRA (Hankinson and Cove, 1974), which suggests that the nitrate induction acts on transcription level. Recently the *A. nidulans* and *A. niger gsd*A genes, encoding G6PD were cloned and characterised (van den Broek *et al.*, 1995,1997). Although the coding regions of the *gsd*A genes exhibit a high degree of sequence conservation, extensive homology is absent from the promoters. The structural dissimilarities between the *Aspergillus gsd*A promoters are consistent with a difference in regulation of expression: Analysis of transcription of the *A. niger gsd*A gene showed that it is neither stimulated by nitrate nor repressed by ammonia. In fact, *gsd*A expression in *A. niger* does not respond to any physiological change leading to an increased NADPH consumption, which suggests that the pentose phosphate pathway in this organism is not the primary source for NADPH (van den Broek, 1997).

To study the involvement of NIRA and AREA in the regulation of *gsd*A expression in *A. nidulans*, *gsd*A promoter-*lac*Z fusions were introduced into wild type *A. nidulans* as well as *nir*A, *are*A and *nia*D mutants. For this analysis we used the pAN923-41B$_{BglII}$ vector (van Gorcom *et al.*, 1986: Punt *et al.*, 1990), thus enabling us to target the *gsd*A promoter constructs to the *arg*B locus.

Materials and methods

Strains, growth conditions and plasmids.

For transformation and propagation of plasmids *E. coli* DH5α (λ$^-$, F$^-$, *end*A1, *hsd*R17 (r_k^- m_k^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, ϕ80Δ*lac*ZM15) (Focus, 1986: Sambrook *et al.*, 1989) was used.

As recipients for the *A. nidulans gsd*A promoter-*E. coli lac*Z constructs the following *A. nidulans* strains were used: SAA236 (*y*A2, *met*H2, *arg*B2, *are*A19), SAA244 (*si*A1, *met*H2, *arg*B2, *xpr*D1), SAA9003 (*y*A1, *pyro*A4, *arg*B2, *nir*A1) and SAA1018 (*y*A2, *bio*A1, *met*H2, *arg*B2, *nia*D15). *A. nidulans* G324 (*w*A3, *y*A2, *bio*A1, *arg*B2, *gal*A1, *s*C12, *ivo*A4) was used as wild type. These strains were kindly provided by dr J. Kinghorn (St. Andrews). For mycelial growth approximately 10^7 conidiospores were used to inoculate 100 ml of minimal medium in 250 ml conical flasks. Minimal medium was composed of 1.5 g/l KH_2PO_4 (pH 6.0), 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl and 0.01% yeast extract. After sterilisation the minimal medium was supplemented with 75 μg/ml methionine, 4 μg/ml biotin and 0.1 μg/ml pyridoxine. D-glucose was added to a final concentration of 50 mM and the nitrogen sources L-proline, NH_4Cl and $NaNO_3$ were used in a final concentration of 10 mM. Cultures were incubated for 21 hours at 30°C in a New Brunswick orbital shaker at 250 rpm. Transformation of *A. nidulans* was performed as previously described (van den Broek, 1997).

The plasmids pAN923-41B$_{BglII}$ (van Gorcom *et al.*, 1986: Punt *et al.*, 1990), pGW514 (van den Broek, 1997) and pBluescript SK$^-$ were used in this study.

Isolation and manipulation of nucleic acids

General manipulations of nucleic acids were performed according to Sambrook *et al.* (1989). Dideoxy-sequencing (Sanger *et al.*, 1977) was performed using *Taq* DNA polymerase sequencing kits (Promega) on ds-templates according to the protocols from the suppliers. Gel purified restriction fragments were labelled by the random primed labelling method (Feinberg and Vogelstein, 1983) as described by Sambrook *et al.* (1989). Chromosomal DNA was isolated from mycelium as previously described (van den Broek, 1997)

Computer analysis

Computer assisted analysis of the DNA sequences was done using the GCG 7.0 software package (Devereux *et al.*, 1984) on a VAX computer.

Oligonucleotides and PCR

M13 reverse primer (5'-AACAGCTATGACCAATG-3'),
primer A (5'-GCGGATCCGACTCGATGCGAGGGTCAGC-3'),
primer B (5'-TAGGATCCGCAATTAAGCAGTCGCCGGG-3') and
primer C (5'-GCGAATTCAGATCTCATCTTATCGGGCGGAATGT-3') were used for the amplification of the *A. nidulans* promoter fragments. PCR reactions (100 µl) contained 0.01 ng of CsCl purified pGW514 plasmid DNA, 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 1 mM of all four dNTP's, 100 pmoles of the appropriate primers and 5U of *Taq* DNA polymerase. The mixture was subjected to 30 cycles of denaturation at 93°C, annealing at 55° and extension at 70°C. The PCR reaction mixture was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1 v/v), once with chloroform/isoamylalcohol (24:1), ethanol precipitated and gel purified. The PCR products were digested with *Bam*HI and *Bgl*II and cloned into the unique *Bam*HI site of the pAN923-41B_{BglII} plasmid. The orientation of the promoter insert in pAN923-41B_{BglII} was determined by restriction analysis. The -742/+3 construct was completely sequenced and was found to be identical to the previously determined *A. nidulans gsd*A promoter sequence (van den Broek, 1997).

Preparation of mycelial protein extracts and enzyme assays

Protein extraction from mycelium and enzyme assays were performed as previously described (van den Broek, 1997). The screening for *lacZ*$^+$ transformants was performed according to Kolar *et al.* (1991).

Results

Construction of A. nidulans gsdA promoter-E. coli lacZ fusions

Three promoter fragments of different length were generated by PCR on the cloned *A. nidulans gsd*A gene. The longest fragment comprises the sequences between -1219 and +3 (A in ATG = +1) and the two other fragments contain the sequences between -742 and +3 and between -236 and +3 (Fig. 1).
In the smallest promoter fragment (-236/+3) *gsd*A box 1 is deleted. The oligonucleotides used as PCR primers, were designed to add a *Bam*HI site to the 5' proximal end of the fragment and a *Bgl*II site downstream of the *gsd*A initiation codon. These restriction sites allowed in frame fusion of the promoter fragments to the ATG-less *lacZ* coding region in the promoter probe vector pAN923-41B_{BglII}. By cloning the amplified fragments in the unique *Bam*HI site of pAN923-41B_{BglII}, the *gsd*A promoter-*lacZ* fusions D1, D2 and D3 were created (Fig.1). The *gsd*A promoter-*lacZ* constructs D1, D2, D3 as well as the promoterless pAN923-41B_{BglII} were introduced into the *A. nidulans* strains G324, SAA236 (*are*A19), SAA244 (*xpr*D1), SAA9003 (*nir*A1) and SAA1018 (*nia*D15). Transformants, containing a single copy of the constructs integrated at the *arg*B locus, were selected by Southern analysis (not shown). With *A. nidulans* SAA1018 (*nia*D15) only multicopy transformants were obtained.

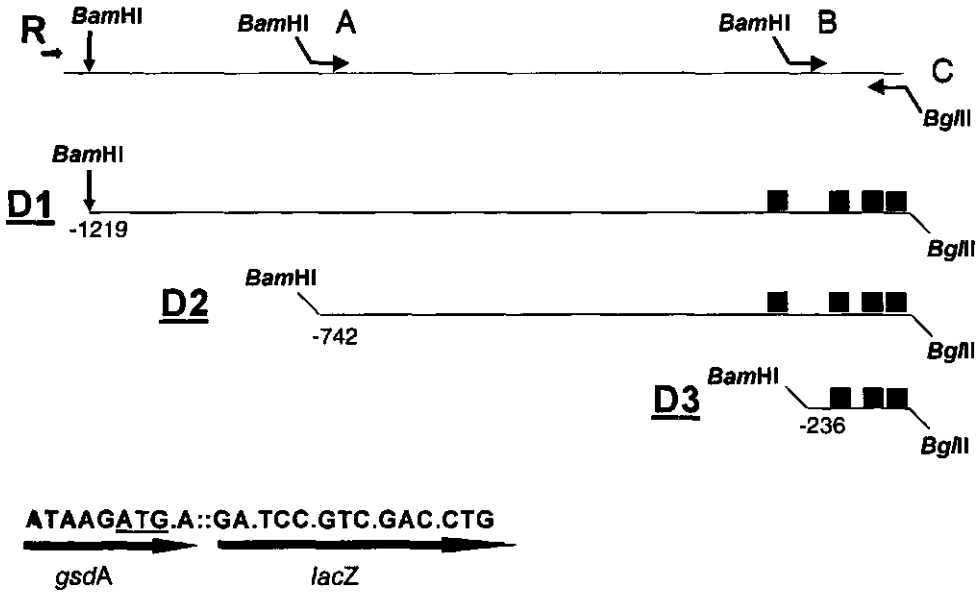

Figure 1. Construction of the *A. nidulans gsd*A promoter-*E. coli lac*Z fusion vectors.
With the M13 reverse sequencing primer (R) and primer C the region between -1230 and +3 (A in ATG = +1) of the *A. nidulans gsd*A promoter was amplified from the plasmid pGW514. This plasmid contains the complete *A. nidulans gsd*A gene (van den Broek *et al.*, 1997). Using an internal *Bam*HI site and the *Bgl*II site in primer C, the 1219 bp of the 5' proximal region and the ATG were fused in frame to the *lac*Z coding region in the promoter probe vector pAN923-41B$_{BglII}$. The resulting plasmid was named pGW547. The nucleotide sequence at the fusion point between the *gsd*A promoter and the *lac*Z reporter gene is shown in the bottom part of the figure. The translational initiation codon is double underlined and the border between the *gsd*A and *lac*Z sequences is marked with double colons. With primer combination A and C the region between -742 and +3 was amplified and with primer combination B and C the region between -236 and +3. The *Bam*HI sites in primers A and B and the *Bgl*II site in primer C were used to clone both PCR products into pAN923-41B$_{BglII}$, resulting in the plasmids pGW549 and pGW550 respectively. For convenience these *A. nidulans gsd*A promoter-*lac*Z fusion constructs will be referred to as D1 (-1219/+3), D2 (-742/+3) and D3 (-236/+3) respectively. The *gsd*A boxes, four sequence motifs that have been conserved between the *A. nidulans* and *A. niger gsd*A promoters, are depicted as black boxes.

The effect of nitrogen sources on G6PD activity in *A. nidulans* transformants

To investigate the influence of the presence of the *gsd*A promoter-*lac*Z fusions on the expression of the resident *gsd*A gene, transformants of all five strains were grown for 21 hours on minimal medium (MM) containing either proline alone, proline and nitrate or proline, nitrate and ammonia as nitrogen source. The G6PD specific activity in protein extracts was determined and the results of these experiments are summarised in figure 2.

In the WT strain, the G6PD enzyme activity of the D1, D2 and D3 transformants on all media is identical to the activity in the transformant containing a promoterless pAB923-41B$_{BglII}$ vector (Fig. 2). This indicates, that there is no gross interference between the resident *gsd*A promoter and the *gsd*A pro-

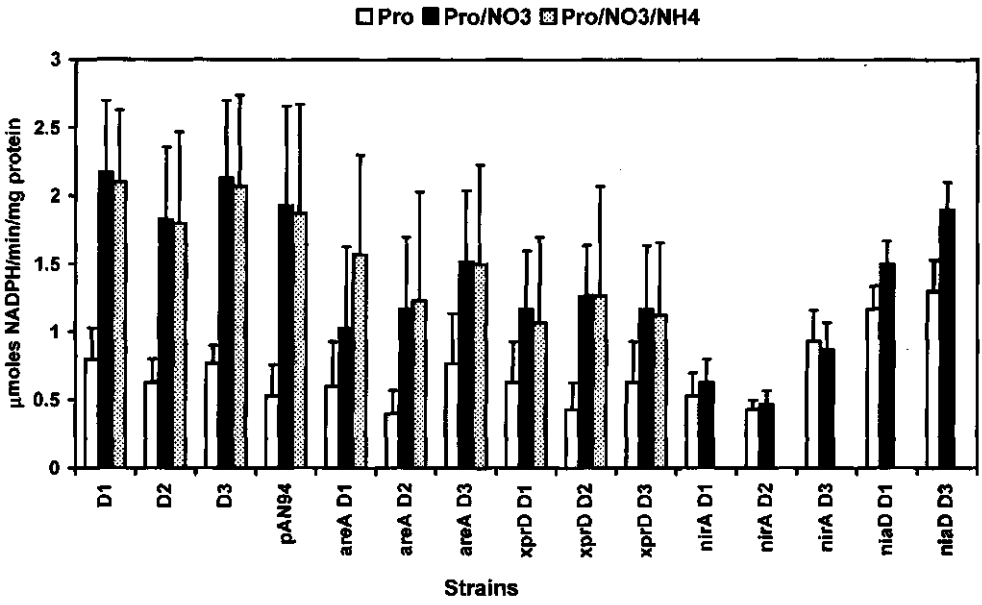

Figure 2. G6PD enzyme activity in *A. nidulans* transformants grown on different nitrogen sources. Single copies of *gsd*A promoter-*lac*Z fusion constructs pGW547 (D1, -1219/+3), pGW549 (D2, -742/+3) and pGW550 (D3, -236/+3) were integrated at the *arg*B locus in the *A. nidulans* strains G324 ('wild type'), SAA9003 (*nir*A1), SAA236 (*are*A19) or SAA244 (*xpr*D1). For *A. nidulans* SAA1018 (*nia*D15) only multicopy transformants were obtained. Only the relevant mutations in these strains are indicated in the graph, for the complete genotypes see the Materials and Methods section. A G324 transformant containing a single copy of the promoter probe vector pAN923-41B$_{argB}$, integrated at the *arg*B locus, is designated in the graph as pAN923 and was used as a control. The transformants were grown for 21 hours at 30°C with either L-proline, a mixture of L-proline and $NaNO_3$ or L-proline, $NaNO_3$ and NH_4Cl as nitrogen source. Immediately after harvesting, protein extracts were made and assayed for G6PD activity. The data in the graph for L-proline and L-proline/NO_3^- are the means of three and for L-proline/NO_3^-/NH_4^+ of two independent experiments. The standard errors are indicated with error bars.

moter-*lac*Z fusions. In the WT background all transformants, irrespective of the transformed promoter construct, show an increased G6PD activity on proline/NO_3^- compared to proline alone. Addition of ammonia, does not reduce G6PD activity in any of the transformants compared to proline/NO_3^- in the WT background. This indicates that G6PD enzyme activity is subject to nitrate induction but not subject to nitrogen metabolite repression.

In the *nir*A1 background no increase in G6PD enzyme activity on proline/NO_3^- in any of the transformants is observed, which indicates that NIRA is essential for nitrate induction. The G6PD enzyme activity on proline of all transformants in the *are*A19 and *xpr*D1 background is virtually identical to the activity in the wild type control. All transformants in the *are*A19 and *xpr*D1 background exhibit a NO_3^--induction of G6PD enzyme activity, albeit the average level of induction is less than in the wild type strain. The presence of ammonia in the medium does not alter the G6PD enzyme activity in either strain compared to the level on proline/NO_3^-. These data indicate that AREA is not essential for the nitrate induction of G6PD enzyme activity and that *gsd*A expression is not subject to nitrogen metabolite repression.

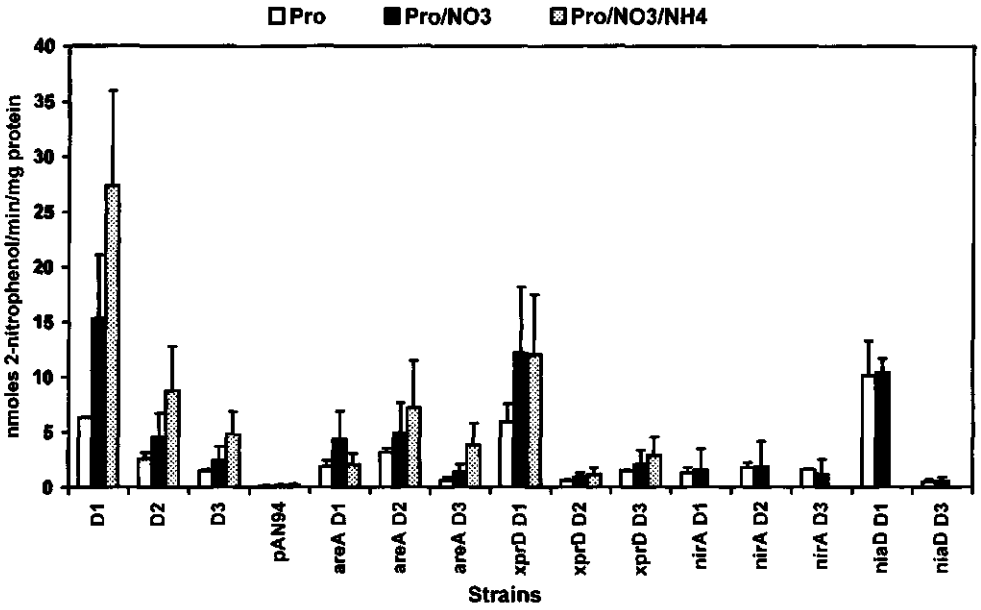

Figure 3. ß-galactosidase activity of *gsd*A promoter-*lac*Z fusions in wild type and nitrate metabolism mutants of *A. nidulans*. Single copies of *gsd*A promoter-*lac*Z fusion constructs D1, D2 and D3 were integrated at the *arg*B locus in *A. nidulans* G324 ('wild type') and SAA9003 (*nir*A1), SAA236 (*are*A19), or SAA244 (*xpr*D1). All *A. nidulans* SAA1018 (*nia*D15) transformants proved to contain multiple copies of the promoter-*lac*Z fusions. Again in this graph only the relevant mutations of these strains are given, for the complete genotype of these strains see the Materials and Methods section. An *A. nidulans* G324 transformant containing a single copy of the promoter probe vector pAN923-41B_{BglII}, designated as pAN923 in the graph, served as a background control. The transformants were grown for 21 hours at 30°C in MM with either L-proline, a mixture of L-proline and $NaNO_3$ or L-proline, $NaNO_3$ and NH_4Cl. Immediately after harvesting, protein extracts were prepared and assayed for ß-galactosidase enzyme activity. The data presented in the graph for L-proline are and L-proline/NO_3^- are the mean of three and for L-proline/NO_3^-/NH_4^+ of two independent experiments. The standard deviations are indicated with error bars.

The G6PD activity in the *nia*D15 mutant on proline is higher than in the WT strain and increases only marginally upon addition of nitrate. In the *nia*D15 mutant, NIRA cannot be inactivated by nitrate reductase and as a consequence this mutant exhibits induced levels of all nitrate utilisation enzymes in the absence of nitrate (Pateman *et al.*, 1964). Therefore, the increased G6PD activity on proline could also represent such an induced level.

*gsd*A promoter deletion analysis in wild type, *nir*A, *nia*D and *are*A mutants

The same protein extracts, used for the G6PD activity assay in the previous section, were also analysed for ß-galactosidase activity. In the WT strain ß-galactosidase activity decreases as more of the *gsd*A promoter is deleted. However, all three constructs exhibit ß-galactosidase activity, well above back-

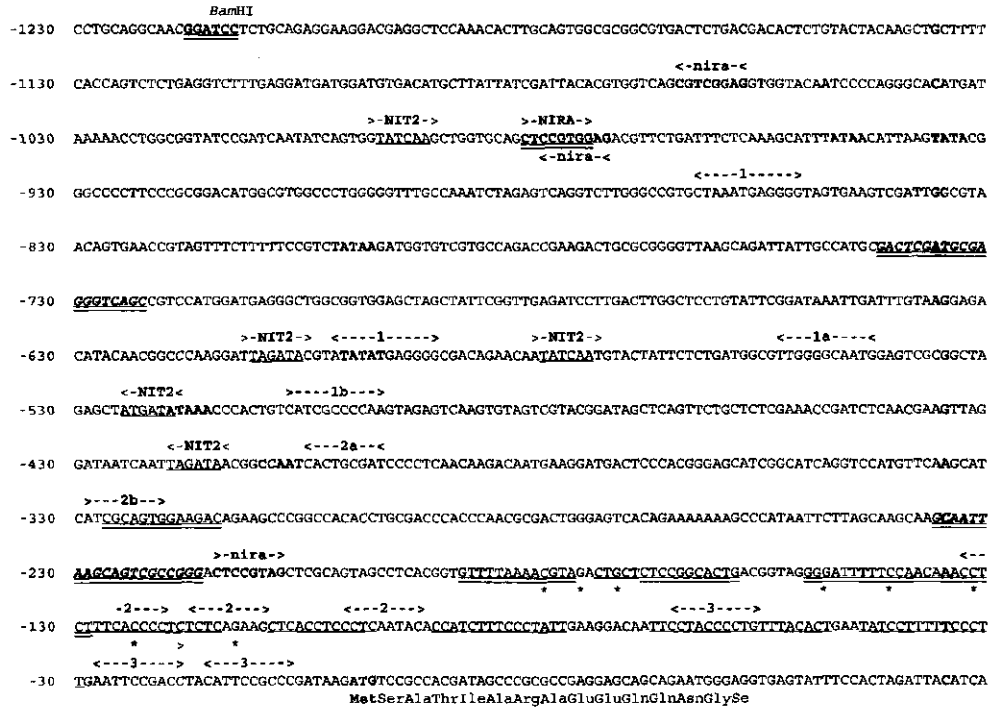

Figure 4. Nucleotide sequence of the *A. nidulans gsd*A promoter.

The *Bam*HI site marks the beginning of the sequence analysed in this study. The positions of the target sequences for the oligonucleotides, used to amplify the promoter fragments, are doubly underlined and marked in bold italic print. The putative CT-boxes are underlined and the *gsd*A boxes 1 to 4 are doubly underlined. The sequences that resemble **TATA** and **CCAAT** box consensus are indicated in bold bold print. All sequences that fit the *N. crassa* NIT2 footprint (Fu and Marzluf, 1990) and the *A. nidulans* NIRA footprint (Punt *et al.*, 1995) or their complement are underlined and marked **NIT2** or **NIRA**. The three sequences that only resemble the NIRA consensus CTCCGHGG or its complement (mismatches are underlined): CGTCGGAG (-1064 to -1057), CCGTGGAG (-1080 to -1073) and CTCCGTAG (-215 to -208) are marked **nira**. The transcription start points are indicated with asterisks (*). Finally, the direct repeats (1, 2 and 3) and the inverted repeats (1a-1b, 2a and 2b) are marked with dashed arrows (>---->).

ground level (Fig. 3). This demonstrates that 57 nucleotides upstream of the 5' proximal transcription start point (Fig. 4) are sufficient to drive *gsd*A transcription. This region does not contain the conserved *gsd*A box 1, which proves that this box is not essential for promoter activity in *A. nidulans*.

In the wild type strain, only the ß-galactosidase activity of the D1 transformant increases significantly on MM with proline/NO_3^- compared to MM with proline alone. In none of the transformants, carrying the *nir*A1 mutation, nitrate induction is observed. These data strongly suggest that the nitrate induced increase in G6PD activity is the result of enhanced *gsd*A transcription. Additionally, these data suggest that this response is mediated by NIRA. Since only promoter construct D1 exhibits the nitrate induction, the nitrate responsive elements should reside in the region between -1219 and the -743. Inspection of the nucleotide sequence of the *A. nidulans gsd*A promoter (Fig. 4) reveals only one sequence (CTCCGTGG, position - 1081 to -1074) that perfectly fits the *A. nidulans* NIRA footprint

(CTCCGHGG, Punt *et al.* 1995). However, in the region between -1219 and -743 there are two sequences (CGTCGGAG, position -1064 to -1057 and CCGTGGAG, position -1080 to -1073) that exhibit a single mismatch to the complement of NIRA consensus and in the region between -236 and +3 (D3) resides another sequence (CTCCGTAG, position -215 to -208) that fits the NIRA footprint except for a single mismatch (Fig. 4).

Apart from the complete absence of nitrate induction in the *nir*A1 background, also the basal level promoter activity of the D1 construct is significantly reduced compared to the same construct in the WT strain. Again this suggests that any NIRA binding sites should be located upstream of position -742. In none of the transformants, addition of ammonia alters ß-galactosidase activity significantly. This is in accordance with the observed absence of any effect on G6PD enzyme activity (Fig. 2) and suggests *gsd*A expression is not subject to nitrogen metabolite repression. Nitrate induction of *gsd*A is observed in the *are*A19 mutant, but the absolute ß-galactosidase activity of D1 is lower than in the WT. By contrast, the ß-galactosidase activity of constructs D2 and D3 on all media in the *are*A19 background resembles their activity in the WT background. On proline and proline/NO_3^-, the *gsd*A promoter activity of the D1 transformant in the *xpr*D1 background is identical to the activity in the WT strain. Despite the almost opposite behaviour of the D1 construct in the *are*A19 and the *xpr*D1 mutants, the G6PD enzyme activity in these transformants (Fig. 2) is almost identical on all media. Construct D2 shows a general decrease in promoter activity in the *xpr*D1 mutant compared to the D2 construct in WT background, but still exhibits WT responses to the nitrogen sources. The ß-galactosidase activities of construct D3 in the *xpr*D1 background on all media are virtually identical to the activities in the wild type background. These data indicate that nitrate induction of *gsd*A transcription is not subject to nitrogen metabolite repression and is not dependent on AREA. Still, AREA seems to be involved in *gsd*A expression and probably exerts its effect on transcription in the region between -1219 and -742.

In the *nia*D15 mutants, the ß-galactosidase activity of the (multicopy) transformants D1 on proline is higher than in the (single copy) WT. The ß-galactosidase activity of D3 in the *nia*D15 mutant is lower as the same construct in the WT. Neither in the D1 nor the D3 transformant an increase in *gsd*A promoter activity is observed upon addition of nitrate. The observed effects can either be the result of the *nia*D15 mutation, which would be in line with the current model, or can be attributed to the multiple copies of D1 or D3, which these transformants contain.

Discussion

In *A. nidulans* the NADPH, which is vital for the operation of the nitrate utilisation pathway, is mainly supplied by the two dehydrogenases from the pentose phosphate pathway G6PD and 6PGD (Hankinson, 1974). To analyse the mechanism of the nitrate induction of G6PD expression, we performed a deletion analysis of the *gsd*A promoter in wild type and *nir*A, *nia*D and *are*A mutant strains. In complete agreement with the results of Hankinson and Cove (1974), we found that growth on nitrate increases G6PD activity and that this nitrate induction is mediated by NIRA. The increased G6PD enzyme activity is not the result of an increased growth rate on nitrate, as reported by Carter and Bull (1969), since the increase is also observed in mutants (e.g. *are*A19), that are unable to metabolise nitrate (Hankinson and Cove, 1974). The *gsd*A promoter-*lac*Z fusions clearly demonstrate that NIRA mediates the nitrate induction of G6PD enzyme activity by stimulating *gsd*A transcription.

Since only the D1 construct exhibits significant nitrate induction, the nitrate responsive elements in the *gsd*A promoter should be localised upstream of position -742. This coincides with the location of the only perfect match (CTCCGTGG, position -1080) with the *A. nidulans* NIRA footprint (CTC-

CGHGG, Punt *et al.*, 1995). The region upstream of -742 contains two other sequences that resemble, but do not perfectly fit, the complement of the NIRA footprint. However, experiments with the *nii*A and *nia*D promoters suggest that only sequences that perfectly match the NIRA consensus CTCCGHGG are functional (Punt *et al.*, 1995).
Therefore, the sequences CGTCGGAG (-1064 to -1057) and CCGTGGAG (-1080 to -1073) do probably not act as functional NIRA sites. This would also explain why the sequence CTCCGTAG (-215 to -208) does not confer nitrate induction to construct D3. Consequently, the *A. nidulans gsd*A promoter contains only a single functional NIRA site, whereas four NIRA binding sites confer nitrate induction to the *A. nidulans nii*A-*nia*D promoter (Punt *et al.*, 1995).

Since *gsd*A transcription in the *are*A19 mutant is still stimulated by nitrate, NIRA is not dependent on AREA for its transcription stimulating activity. This means that, at least for *gsd*A transcription, there is no necessity for a cooperation or interaction between NIRA and AREA as suggested by the phenotype of the *nir*A^{cld}101 mutant (Rand and Arst, 1978).

Furthermore, our data are not consistent with a model whereby NIRA merely stimulates *gsd*A transcription on nitrate. For in a *nir*A1 mutant *gsd*A transcription is reduced under non-inducing conditions (proline or urea) compared to the WT.
Careful reexamination of the data of Hankinson and Cove (1974), showed that they also measured this reduced G6PD activity in the *nir*A mutant. The current model for the regulation of the nitrate utilisation genes (Cove, 1979), implies a low steady state level of NIRA under non-inducing conditions. Therefore, the decreased level of *gsd*A transcription in the *nir*A1 mutant could be explained by assuming that latter non-inducing steady state level of NIRA is important for normal *gsd*A transcription. The fact that only the non-induced transcription level of D1 is affected in the *nir*A1 mutant, suggests that NIRA stimulates transcription under non-inducing conditions from a position upstream of position -742.

From the ß-galactosidase activities in the *are*A19 and *xpr*D1 mutants, it is clear that AREA affects the *gsd*A transcription. Our data suggest that the region between -1219 and -742 contains AREA responsive elements. Since the AREA effect can be attributed to a specific domain in the *gsd*A promoter-*lac*Z fusions, it is unlikely to be an artefact from the integration at the *arg*B locus. Since the *are*A19 mutant does not express nitrate reductase (Hawker *et al.*, 1992), NIRA should not be inactivated and according to the current model one would expect induced levels of *gsd*A transcription even in the absence of nitrate. However, we find that *gsd*A transcription in the *are*A19 mutant is reduced compared to WT and still nitrate-inducible. According to the same model, the *xpr*D1 mutation should not have any effect on *gsd*A transcription, because its expression is insensitive to nitrogen metabolite repression. Still, the promoter activity of D2 in the *xpr*D1 mutant differs from the activity of D2 in the wild type strain. Therefore, we have to devise a model that reconciles the contradicting observations that *gsd*A expression is not subject to nitrogen metabolite repression, but its transcription is still regulated by AREA. The observed effects could be the result of transcriptional regulation of the *are*A gene, because under repressing conditions longer *are*A transcripts have been observed. These could result in an AREA protein with altered characteristics (Caddick, 1992), that still could stimulate transcription of the *gsd*A gene under repressing conditions.

Inspection of the *A. nidulans gsd*A promoter shows that both the -1219/-741 and the -742/-236 region (Fig. 4) contain sequences that are either identical or complementary to the consensus *N. crassa* NIT2 footprint (Fu and Marzluf, 1990). Some of the sequences in the region -742/-236 fit the NIT2 consensus perfectly, despite that AREA exerts its effect on transcription upstream of position -742. Illegitimate binding of the truncated AREA protein, encoded by *xpr*D1 (Arst, 1989), to latter putative AREA binding sites might be the cause of the inhibition of promoter activity of the D2 construct in that mutant. This of course implies cooperative effects in the correct assembly of the transcription complex of

the *gsd*A promoter. Since AREA affects the activity of a large number of genes, it can not be excluded that the changes in *gsd*A transcription in the *are*A19 and *xpr*D1 mutants are the indirect result of a more general change in the cellular metabolism. Bandshift assays on the *gsd*A promoter in WT, the *are*A19 and the *xpr*D1 mutant, both under inducing and repressing conditions, would provide a definite answer whether AREA actually binds or not.

Considering the fact that *gsd*A expression is not subject to nitrogen metabolite repression, we have to address the significance of the AREA regulation. It has been suggested that AREA not only mediates nitrogen metabolite repression but also oxygen repression (Shaffer *et al.*, 1988) in the expression of an asparaginase gene. Oxygen repression might also explain the presence of AREA regulation of the *gsd*A promoter. Low oxygen levels induce nitrate and nitrite reductase as well as increase the level of G6PD activity in *A. nidulans* (Carter and Bull, 1969). At low oxygen concentrations, nitrate serves as a terminal electron acceptor while the pentose phosphate pathway serves as a bypass of the glycolysis. In this way the fungus probably lowers the production of NADH, while using nitrate as a dump for excess NADPH (Carter and Bull, 1969).

In a number of cases the G6PD enzyme activity, reflecting the expression of the resident *gsd*A gene, does not match the transcription activity of the *gsd*A promoter *lac*Z fusion. The promoter activity of D1 in the *nir*A1 and *are*A19 mutants is much lower than in the *xpr*D1 and WT background, yet their G6PD activities show only minor deviation from the WT level. This discrepancy cannot be caused by expression of a second G6PD encoding gene, since no sequences that cross-hybridise to *gsd*A are detected in *A. nidulans* chromosomal DNA. It could however, be an artefact of integration at the *arg*B locus or an incomplete *gsd*A promoter in the reporter construct. Also post-transcriptional regulation of *gsd*A expression by enhanced G6PD enzyme or *gsd*A mRNA stability in the *nir*A1 and *are*A19 mutant cannot be ruled out.

Although the resolution of this *A. nidulans gsd*A promoter analysis does not allow detailed comparison with the *A. niger gsd*A promoter, the data give sufficient evidence for the different regulation of the two *Aspergillus gsd*A genes. *A. niger gsd*A expression is not stimulated by nitrate, but *A. nidulans gsd*A transcription clearly is. The latter suggests that in *A. niger*, *nir*A is not involved in the regulation of *gsd*A expression. Inspection of its nucleotide sequence (van den Broek *et al.*, 1997) revealed that sequences that fit the *A. nidulans* NIRA footprint are absent from the *A. niger gsd*A promoter. The fact that NIRA affects not only the nitrate induction but also the uninduced level of the *A. nidulans gsd*A gene, again suggests that the *A. niger* promoter must function differently. Furthermore, when *gsd*A box 1 and all the upstream sequences are deleted from the *A. nidulans* promoter, it still retains almost 25% of its original transcription level, yet an equivalent deletion in the *A. niger gsd*A promoter reduces transcription to background level (van den Broek, 1997). The latter strongly suggests that the regions containing the *gsd*A boxes are functionally different in *A. nidulans* and *A. niger*. To investigate the role of these boxes in *gsd*A transcription, specific deletion or mutagenesis of the *gsd*A boxes in both species will be necessary.

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Summary

Glucose-6-phosphate (G6P) is a central metabolite, that can either be metabolised via the glycolytic and tricarboxylic acid cycle to generate ATP, or converted into storage molecules or can be directed to the pentose phosphate pathway to yield NADPH and various pentoses. This thesis focuses on one of these G6P consuming reactions, catalysed by glucose-6-phosphate dehydrogenase (G6PD), in which G6P is oxidised and $NADP^+$ acts as electron acceptor. The central theme of this thesis is the role of glucose-6-phosphate dehydrogenase (G6PD) enzyme activity in the generation of the cytosolic NADPH pool. This cytosolic NADPH pool serves as the reducing agent in a large number of biosynthetic reactions, in the protection against oxidation damage and in the utilisation of nitrate and certain pentoses. Only a limited number of catabolic reactions generate NADPH of which the one catalysed by G6PD is usually considered to be the most important.

If G6PD were the major producer of NADPH, one would expect 1) G6PD enzyme activity to respond to changes in NADPH demand and 2) $G6PD^-$ mutations to be extremely deleterious or even lethal. According to these criteria, examples can be found in the scientific literature, where G6PD appears to be either vital for cytosolic NADPH production or appears to be completely superfluous. *Aspergillus nidulans* and *Aspergillus niger* were chosen as model systems because of their ability to metabolise a great variety of carbon- and nitrogen sources and their well developed genetics. These characteristics would allow us to use nitrogen- and carbon sources that influence NADPH consumption and to employ molecular biological techniques to study and manipulate the regulation of G6PD expression.

The biochemical characteristics of the purified G6PD enzymes from *A. nidulans* and *A. niger* suggest the involvement of these G6PD enzymes in NADPH production. For both pure enzyme preparations, the main modulator of G6PD enzyme activity is the redox potential as is reflected by its stimulation by $NADP^+$ and its competitive inhibition by NADPH. Additionally, these observations show that at least part of the regulation of G6PD activity in *A. nidulans* and *A. niger* is exerted at the enzyme level. In their biochemical characteristics, their primary and secondary structure the G6PDs from *A. nidulans* and *A. niger* are virtually identical. Both fungal G6PD enzymes bind their substrates G6P and $NADP^+$ in a random order and exhibit a strict specificity towards them. Whether the presence of two proteins in both pure *Aspergillus* G6PD preparations, has any physiological significance or is just an artefact due to proteolytic degradation of the native G6PD during the purification procedure, remains to be determined. However, the cloning of the G6PD encoding genes does provide a clue to the origin of the two G6PD proteins. In *A. nidulans* both G6PD proteins should be derived from the *gsd*A gene, since only a single G6PD encoding gene could be detected on genomic Southern blots. For the two *A. niger* G6PD proteins, the situation is less clear because genomic Southern blots revealed the presence of additional DNA bands that cross-hybridised strongly to the *gsd*A gene and which could represent a second G6PD encoding gene.

The *gsd*A genes from *A. nidulans* and *A. niger* have an identical structure; both contain nine introns, which are located at exactly equivalent positions with respect to the coding region. Furthermore, both genes exhibit strong DNA sequence homology in the coding region, but in the introns, 5'- and 3'-flanking sequences, this homology drops significantly. Alignment of the deduced *gsd*A amino acid sequences with other eukaryotic G6PDs reveals strong homology at the amino acid level and allows localisation of important domains like the putative catalytic site and $NADP^+$ bindings sites. The cloning of the *gsd*A genes allows us to manipulate the G6PD activity directly. The increase of G6PD activity by

the introduction of multiple copies of the *gsd*A gene results in *A. niger* in grossly disturbed growth especially on media containing reduced nitrogen sources. The degree of growth inhibition and the increase in G6PD activity are directly related to the number of functionally integrated *gsd*A genes. This observation suggests the reduced growth is a direct consequence of the increased G6PD activity. One can explain these phenomena by assuming that increased G6PD activity results in overproduction of NADPH. If for this excess of NADPH no acceptor is available (e.g. nitrate), the redox potential is disturbed and growth is inhibited. A similar experiment with the *A. nidulans* gene resulted in cotransformants with a single additional *gsd*A copy, that only slightly overexpressed G6PD end did not exhibit reduced growth.

This already shows that despite the similarity in the biochemical characteristics and in primary structure of the enzymes and genes, there are remarkable differences in the regulation of the *A. niger* and *A. nidulans gsd*A expression. In *A. nidulans*, but not in *A. niger*, G6PD enzyme activity and *gsd*A transcription respond to the increased NADPH demand during growth on nitrate. These observations indicate that in *A. nidulans* G6PD does play an important role in NADPH production, whereas in *A. niger* it probably does not. Further evidence for this statement comes from the observation that in *A. niger* G6PD enzyme activity and steady state mRNA levels do not change in response to growth on xylose or in response to oxidation stress.

The homology in the *gsd*A 5'-upstream regions is limited to four 11 to 22 bp long sequence blocks, named *gsd*A-boxes, which in both promoters appear in the same order. Homologues of *gsd*A-boxes 1, 2 and 4 are also encountered in the same order in the *S. cerevisiae zwf*1 promoter. In both Aspergilli, the *gsd*A-box region contains all transcription start sites: in the case of *A. niger* there are four and in *A. nidulans* there are as much as eight. We did not find any conservation, neither in the number of transcription sites nor the site of initiation. In *A. niger* all deletions within the region containing the *gsd*A-boxes, reduce transcription to background level while such a deletion in *A. nidulans* retains 25% of its original transcription. These observations indicate that despite the *gsd*A-box homology, these regions still differ functionally.

From the deletion study of the *A. nidulans gsd*A promoter, it is clear that the nitrate induced increase in G6PD activity is caused by an increased *gsd*A transcription. Furthermore, evidence has been obtained that the NIRA transcription factor, which mediates induction of the nitrate utilisation pathway, is also involved in the nitrate stimulation of *gsd*A transcription in *A. nidulans*. Furthermore our data show clearly, that NIRA plays an important role in the regulation of *gsd*A transcription in the absence of nitrate. This phenomenon is probably yet another difference in the regulation of *gsd*A expression between *A. nidulans* and *A. niger*. Since *A. niger gsd*A expression does not respond to nitrate induction, it is unlikely that the uninduced level of its *gsd*A transcription is under the control of NIRA. This NIRA dependence of *gsd*A transcription might also explain the remarkably different behaviour of the two *gsd*A genes in cotransformation experiments.

In *A. nidulans*, titration of the NIRA protein by the cotransformed *gsd*A gene might limit the expression of the cotransformed *gsd*A copies. At the same time cotransformants with multiple copies of *gsd*A would be lost on the nitrate containing selection medium, since NIRA titration would render them incapable of nitrate utilisation. These two phenomena would explain the low cotransformation frequency of the *A. nidulans gsd*A gene and low G6PD overproduction observed in *A. nidulans* cotransformants. Conversely, the absence of NIRA regulation in the *gsd*A expression in *A. niger* does not limit overproduction of G6PD in *gsd*A cotransformants. Furthermore, the absence of NIRA titration in *A. niger gsd*A cotransformants allows the use of nitrate as an acceptor for the excess NADPH.

In contrast to the other members of the nitrate utilisation regulon (e.g. *nia*D, *nii*A and *crn*A), is *gsd*A expression not subject to nitrogen catabolite repression exerted by the *are*A gene product. Still,

AREA seems to act on the *A. nidulans gsd*A promoter. The physiological significance of this phenomenon remains to be established.

The data presented in this thesis show that although the primary structure and biochemical characteristics of an enzyme from two different organisms are virtually identical, there can be significant differences in the regulation of their expression and hence in their physiological function. Of course our data provide only an outline of the regulation of G6PD activity in two Aspergilli and, unfortunately, raise more questions then they answer.

Our data indicate that G6PD does not play a crucial role in the generation of the cytoplasmic NADPH pool in *A. niger*. What is then the physiological function of G6PD enzyme activity in *A. niger*? Furthermore, as it is difficult to assign a physiological function to the *A. niger gsd*A gene, is it reasonable to assume that the fragments that cross-hybridise to the *gsd*A gene in digests of *A. niger* genomic DNA could represent a second G6PD encoding gene? Clearly, the construction and characterisation of a *gsd*A null mutant should provide important clues to these questions. Since G6PD is not the main producer of NADPH in *A. niger*, a *gsd*A null mutation should be viable. This of course raises the question which pathway provides the bulk of the cytoplasmic NADPH in *A. niger*?

In *A. nidulans* G6PD enzyme activity is clearly involved in the maintenance of a proper cytoplasmic $NADP^+$/NADPH ratio as its expression responds to the increased NADPH consumption during growth on nitrate. From this observation we would predict that a *gsd*A null mutant, if not lethal, would be either unable to utilise nitrate or would exhibit reduced growth on this nitrogen source. However, the fact that no G6PD mutants have been found among the nitrate non-utilising mutants suggests that such mutations are in fact lethal. How does *A. nidulans gsd*A expression respond to NADPH consuming processes other than nitrate utilisation? Are these responses also regulated at the transcriptional level and if so which transacting factors are involved? What is the function of the individual *gsd*A-boxes in *A. niger* and *A. nidulans*? Why do the regions containing the *gsd*A-boxes differ functionally, despite the obvious homology? Does titration of NIRA rely occur in *A. nidulans gsd*A cotransformants? In any case these questions demonstrate the necessity for further research!

Samenvatting

Men kan de celstofwisseling op vatten als een netwerk van chemische reacties, die vrijwel allemaal gekatalyseerd worden door enzymen. Deze chemische reacties genereren energie, bouwstenen en reservestoffen. Glucose-6-fosfaat (G6P) is een centrale verbinding in de celstofwisseling: G6P kan via de glycolyse, de citroenzuurcyclus en de elektronentransportketen omgezet worden in energie of het kan opgeslagen worden in de vorm van glycogeen of zetmeel. Bovendien kan G6P via de pentosefosfaat cyclus omgezet worden in NADPH en C5 suikers (pentoses). Onderwerp van dit proefschrift is het enzym glucose-6-fosfaat dehydrogenase (G6PD) en zijn rol in de celstofwisseling. Het G6PD katalyseert de eerste reactie van de pentosefosfaat cyclus. Samen met 6-fosfogluconaat dehydrogenase (6PGD) vormt G6PD het oxydatieve gedeelte van de pentosefosfaat cyclus, waarin glucose (druivesuiker) met behulp van NADP+ wordt geoxydeerd tot ribulose-5-fosfaat.

In het niet-oxydative gedeelte van de pentosefosfaat cyclus worden uit het ribulose-5-fosfaat C3, C4, C5 en C6 suikerfosfaat verbindingen gemaakt die o.a. nodig zijn voor de synthese van nucleotiden en aromatische aminozuren. Aangezien deze laatste verbindingen ook vanuit de glycolyse kunnen worden aangemaakt, kan NADPH als het belangrijkste reactie produkt van de pentosefosfaat cyclus beschouwd worden. Dit NADPH is essentieel voor de aanmaak van een groot aantal belangrijke verbindingen en speelt een belangrijke rol bij de bescherming tegen oxydatie schade. Ook tijdens de groei op nitraat en bepaalde C5 suikers is NADPH onmisbaar. Er is echter slechts een klein aantal enzymen bekend, dat NADPH genereert. Wanneer glucose als enige koolstofbron wordt gebruikt, dan zijn de specifieke activiteiten van G6PD en 6PGD het hoogst van al de NADPH producerende enzymen. Dit suggereert dat, tijdens groei op glucose, G6PD en 6PGD de meest belangrijke NADPH producenten zijn.

Indien dat laatste waar is, dan zou je mogen verwachten dat: 1) de activiteit van G6PD en 6PGD zou variëren afhankelijk van de behoefte aan NADPH en 2) G6PD- mutaties ernstige consequenties zouden hebben of zelfs letaal zouden zijn.

Als we uitgaan van deze twee criteria, dan vinden we in de wetenschappelijke literatuur tegenstrijdige aanwijzingen voor het belang van G6PD in de NADPH produktie. Bij coli-bacteriën, bakkersgist en fruitvlieg lijken G6PD mutaties nauwelijks effect te hebben. Bij mensen blijven de gevolgen van G6PD- mutaties beperkt tot de erythrocyten. Uit deze waarnemingen zou men kunnen afleiden, dat de rol van G6PD bij de produktie van NADPH beperkt is. Toch zijn er in coli-bacteriën, rat en mens duidelijke aanwijzingen dat verhoging van het NADPH verbruik leidt tot verhoging van de G6PD activiteit.

In dit proefschrift hebben we ervoor gekozen om de regulatie van de G6PD activiteit te bestuderen in *Aspergilus nidulans* en *Aspergillus niger*. Omdat deze twee filamenteuze schimmels kunnen groeien op een groot aantal koolstof en stikstofbronnen, worden ze vaak gebruikt voor stofwisselingsstudies. De metabole flexibiliteit van deze schimmels laat het toe groei omstandigheden te kiezen die verschillen in NADPH-behoefte. Door te bestuderen hoe de G6PD activiteit reageert op die veranderingen in NADPH consumptie, kan men een indruk krijgen van het belang van G6PD voor de NADPH productie. Bovendien kan, men met behulp van moleculair biologische technieken, de regulatie van de G6PD activiteit direct op gen niveau te bestuderen en zelfs gericht manipuleren.

Omdat de biochemische eigenschappen van een enzym al belangrijke aanwijzingen geven over zijn functie in de stofwisseling, is begonnen met de zuivering en karakterisatie van de G6PD enzymen van *A. nidulans* en *A. niger*. De biochemisch eigenschappen van het *A. nidulans* en *A. niger* G6PD blijken

vrijwel identiek te zijn en komen overeen met die van een enzym dat een rol speelt bij de produktie van NADPH. In beide gevallen wordt het enzym gestimuleerd door NADP+ en geremd door NADPH. Zowel het *A. nidulans* als het *A. niger* G6PD enzym vertoont een vrijwel absolute specificiteit met betrekking tot G6P en NADP+. Andere verbindingen, waarvan bekend is dat ze de activiteit van G6PD enzymen uit andere organismen moduleren, blijken echter nauwelijks invloed op de Aspergillus G6PD enzymen te hebben.

In het gezuiverde enzym preparaat van beide Aspergilli blijken echter niet één maar twee G6PD enzymen aanwezig te zijn, die verschillen in molecuul gewicht en isoelectrisch punt. Door de isolatie van het voor G6PD coderende gen (*gsd*A), kon worden vastgesteld dat de beide G6PD enzymen in het *A. nidulans* enzym preparaat door één en hetzelfde gen gecodeerd worden. *A. nidulans* blijkt namelijk slechts één *gsd* gen te bezitten. Echter voor de twee G6PD enzymen in het *A. niger* preparaat is er nog een tweede mogelijke verklaring: Een tweede voor G6PD coderend gen. In genomische Southern blots van *A. niger* kruishybridiseren fragmenten, die niet van het *gsd*A gen afkomstig kunnen zijn. We kunnen daarom niet uitsluiten, dat deze kruishybridiserende fragmenten afkomstig zijn van een tweede G6PD gen.

De structuur van de *A.niger* en *A. nidulans gsd*A genen is vrijwel identiek: beide genen worden door negen introns onderbroken op equivalente posities in het coderend gebied. In het coderend gebied is de homologie op DNA niveau hoog, maar deze daalt in de introns en in de 5' en 3' niet coderende gebieden. De aminozuur-sequenties van beide Aspergillus *gsd*A genen vertonen niet alleen sterke overeenkomst met elkaar, maar ook met die van andere eukaryote G6PD enzymen. Door biochemische informatie te combineren met de waargenomen homologie op aminozuurniveau, kunnen zowel in het *A. nidulans* als in het *A. niger* G6PD het katalytische domein en de NADP+-bindingsplaats gelokaliseerd worden.

Het kloneren van de *gsd*A genen opent de mogelijkheid om de G6PD activiteit gericht te manipuleren en het effect daarvan op het metabolisme te bestuderen. Verhoging van de G6PD activiteit door introductie van extra kopieën van het functionele *gsd*A gen leidt in *A. niger* tot een ernstig verstoorde groei, met name op gereduceerde stikstofbronnen. De waarneming dat de groeiremming en de gemeten G6PD activiteit direct gerelateerd zijn aan het aantal functioneel geïntegreerde *gsd*A kopieën, suggereert dat de verstoorde groei het directe gevolg is van verhoogde G6PD activiteit. Deze waarneming kan verklaard worden door aan te nemen dat de overproduktie van G6PD leidt tot een overproduktie van NADPH. Indien er voor deze overmaat aan NADPH geen acceptor is (zoals bijvoorbeeld nitraat) wordt de redox potentiaal van de cel ernstig verstoord en treedt groeiremming op. Een overeenkomstig experiment met het *A. nidulans gsd*A gen levert alleen cotransformanten op met slechts één extra kopie van het *gsd*A gen. Deze cotransformanten vertonen slechts geringe overproductie van G6PD en groeien normaal. Dit duidt er al op dat er belangrijke verschillen lijken te bestaan in de regulatie van beide *gsd*A genen, ondanks de grote overeenkomsten in biochemische eigenschappen van de enzymen, in de primaire structuur van de monomeren en in de structuur van het genen. Wanneer *A. nidulans* nitraat als stikstofbron gebruikt, reageert de *gsd*A expressie op de toegenomen behoefte aan NADPH. Bij *A. niger* is dat echter niet het geval. Hieruit kan worden afgeleid, dat in *A. nidulans* G6PD een belangrijke rol speelt bij de NADPH produktie, terwijl dat in *A. niger* waarschijnlijk niet het geval is. Deze bewering wordt ondersteund door de waarneming dat noch de G6PD activiteit, noch het *gsd*A mRNA niveau, noch de *gsd*A transcriptie stijgen tijdens groei op D-xylose of als gevolg van oxydatie stress. Blijkbaar wordt in *A. niger* het grootste deel van het NADPH niet in de pentosefosfaat cyclus, maar door een andere stofwisselingsroute geproduceerd. Een alternatief voor het oxydatieve deel van de pentosefosfaat route in *A. niger*, zou de mannitol shuttle kunnen zijn. In deze stofwisselingsroute worden reductie equivalenten van NADH overgedragen op NADP+

De verschillen in expressie tussen het *A. niger* en *A. nidulans gsd*A komen ook tot uiting in de afwezigheid van grote sequentie overeenkomst in het promoter gebied van beide genen. In feite is de homologie beperkt tot vier korte sequentie motieven (11 tot 22 base paren (bp) lang), die in beide genen in dezelfde volgorde aanwezig zijn. Verwante sequenties worden ook in de promoter van het G6PD gen (*zwf1*) van *Saccharomyces cerevisiae* aangetroffen. Ondanks de aanwezigheid van de vier *gsd*A-boxen, blijken de gebieden waarin deze zich bevinden toch niet functioneel identiek te zijn. Deleties in het *gsd*A-box gebied van de *A. niger* promoter reduceren de promoter activiteit tot nul, terwijl een gelijkwaardige deletie in de *A. nidulans gsd*A promoter nog steeds 25% van de oorspronkelijke activiteit vertoont. Mogelijk bevatten de niet-homologe promoterdomeinen, essentiële elementen voor de transcriptie van het *A. niger* gen. In beide promotoren bevinden zich alle transcriptiestarts in het gebied met de vier *gsd*A-boxen, maar er is geen enkele overeenkomst in de posities van de transcriptie initiatie plaatsen. Voorts heeft *A. niger* vier transcriptiestarts en *A. nidulans* acht.Tijdens groei op xylose blijven in *A. niger* de G6PD enzym activiteit en *gsd*A mRNA niveaus gelijk aan die tijdens groei op glucose. Echter de *gsd*A transcriptie echter daalt tot 50% van het niveau op glucose. Mogelijk is dit het gevolg van de inductie van het tweede G6PD gen, gecodeerd door de kruishybridiserende fragmenten.

De nitraat-geïnduceerde verhoging van de G6PD activiteit in *A. nidulans*, is het gevolg van een stimulering van de transcriptie van het *gsd*A gen. Deze transcriptie stimulatie, blijkt afhankelijk van het *nir*A gen produkt (NIRA). Dit NIRA mediëert ook de nitraat-inductie van de andere genen uit het nitraatregulon (zoals *nia*D, *nii*A en *crn*A). Ook het niet-geïnduceerde transcriptieniveau van het *A. nidulans gsd*A gen blijkt afhankelijk te zijn van NIRA. Dat laatste zou wel eens de oorzaak kunnen zijn van het verschillend gedrag van de twee *gsd*A genen in cotransformatie experimenten. Het huidige model voor de regulatie van het nitraatmetabolisme veronderstelt, dat het steady-state niveau van NIRA limiterend is voor de inductie van het nitraatregulon. Echter, deze geringe hoeveelheid NIRA zou dan ook de transcriptie van de extra *gsd*A kopieën beperken, zodat G6PD overproduktie in *A. nidulans gsd*A cotransformanten nauwelijks optreedt. Mogelijk verhindert titratie van NIRA door de geïntroduceerde *gsd*A kopieën ook de inductie van *nia*D en *nii*A in cotransformanten, waardoor deze op onze nitraat selectieplaten niet kunnen groeien. Aangezien in *A. niger* de *gsd*A expressie waarschijnlijk niet NIRA afhankelijk is, treden noch NIRA limitatie van *gsd*A transcriptie, noch NIRA titratie op en dus kan *gsd*A cotransformatie tot G6PD overproduktie leiden. Door de afwezigheid van NIRA titratie in *A. niger gsd*A cotransformanten, kan het nitraatmetabolisme zonder problemen geïnduceerd worden, waardoor het nitraat als acceptor kan dienen voor de geproduceerde overmaat aan NADPH.

In tegenstelling tot de andere nitraatgenen is *gsd*A expressie niet onderhevig aan stikstofmetaboliet-repressie. Vreemd genoeg blijkt de positieve regulator AREA, verantwoordelijk voor stikstofmetaboliet-repressie, wel invloed te hebben op de activiteit van de *gsd*A promoter. Voor dit fenomeen hebben we vooralsnog geen verklaring. Aangezien AREA de expressie van een groot aantal genen reguleert, kunnen we niet uitsluiten dat de effecten van AREA op de *A. nidulans gsd*A promoter het indirecte gevolg zijn van een globale verandering in het cel metabolisme.

De in dit proefschrift gepresenteerde data laten zien, dat sterk homologe genen, zelfs uit nauw verwante organismen, volledig verschillend gereguleerd kunnen zijn. De data in dit boekje vormen slechts een ruwe schets van de regulatie van G6PD activiteit in twee Aspergilli en roepen nieuwe vragen op. Zo suggereren onze data bijvoorbeeld dat G6PD in *A. niger* niet de belangrijkste NADPH leverancier. Dit roept meteen de vraag op wat dan wel zijn fysiologische functie is. Welke stofwisselingsroute levert dan wel het grootste deel van het NADPH? Als het al moeilijk is om een fysiologische functie toe te schrijven aan het *A. niger gsd*A, is het dan waarschijnlijk dat de kruishybridiserende DNA fragmenten een G6PD isozym coderen? Constructie en karakterisatie van een *A. niger gsd*A nul-mutant zou het beantwoorden van deze vragen vergemakkelijken.

In *A. nidulans* speelt G6PD wel een belangrijke rol in de handhaving van cytoplasmatische NADP+/ NADPH balans, zoals blijkt uit de toename in *gsd*A expressie op nitraat. Reageert *gsd*A expressie in *A. nidulans* ook op ander NADPH consumerende processen zoals oxydatie stress of groei op D-xylose? Wordt de repons op deze stimuli ook op transcriptieniveau gereguleerd en wat zijn dan de daarvoor verantwoordelijke transcriptiefactoren? Wat is de functie van de individuele *gsd*A-boxen in *A. nidulans* en *A. niger* en wat veroorzaakt de functionele verschillen? Treedt er bij cotransformatie experimenten met het *A. nidulans gsd*A gen echt titratie van NIRA op? Kortom, voldoende argumenten voor verder onderzoek!

Curriculum vitae

Petrus Johannes Maria van den Broek werd op 22 februari 1961 te 's-Hertogenbosch geboren, waar hij van 1974 tot 1980 het Jeroen Bosch College bezocht. Na het eindexamen Gymnasium β, begon hij zijn biologie studie (oude stijl) aan de Rijks Universiteit Utrecht. In 1983 behaalde hij het kandidaats examen B4 en in 1987 het doctoraal examen met als hoofdvak moleculaire genetica (dr. S.A. Langeveld), als bijvakken moleculaire biologie (dr. H. Amesz) en fysiologische chemie (prof.dr.ir. J.S. Sussenbach) en als nevenrichting biochemie. Van september 1987 tot november 1991 was hij aangesteld als assistent in opleiding bij de vakgroep Erfelijkheidsleer van de Landbouwuniversiteit Wageningen waar hij onder begeleiding van dr. H.W.J. van den Broek en dr. T. Goosen werkte aan dit proefschrift. Vanaf september 1993 werkte hij in het Yeast and Fungal Fermentation Team van dr. P. Niederberger op het Centre de Recherche Nestlé in Lausanne, Zwitserland. Op 1 mei 1995 kwam hij daar in vaste dienst.